Detection and Characterisation of Picobirnaviruses in Human and Rabbit Faeces

Thesis

UNRESTRICTED

DETECTION AND CHARACTERISATION OF PICOBIRNAVIRUSES

IN HUMAN AND RABBIT FAECES

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DEDICATION

To my wife Christine

and my parents

ABSTRACT

Human faecal samples were screened by nucleic acid extraction and polyacrylamide gel electrophoresis (PAGE) for the presence of novel dsRNA viruses. A genome typical of a bisegmented dsRNA virus termed picobirnavirus (PBV) was detected in 5 to 15% of about one thousand human faecal samples. The PBV genome consists of two equimolar segments of dsRNA of approximately 2.45 and 1.75 Kilo base pairs (Kbp) in size. During studies attempting to pass a human PBV (HPBV) strain into rabbits, a coincidental rabbit PBV (RPBV) strain was detected, and subsequently three further unique rabbit strains were detected in newly weaned rabbits. An atypical PBV (APBV) associated with *Cryptosporidium* positive human faecal samples was detected in 20/54 (37%) of samples. The genome of the APBV was 1.75 and 1.55 Kbp in size and all strains detected were virtually identical.

Direct electron microscopy was performed on human faecal samples and small round featureless PBV-like particles with a diameter of 35-40nm were seen. Rabbit PBV-like particles were also detected in PAGE-positive CsCl gradient fractions and peaked at a buoyant density of 1.395 g/ml was obtained.

An immune response temporally associated with virus excretion was demonstrated in one rabbit by immune electron microscopy. Two antigenically and genomically different strains were shown to be circulating in rabbits.

Epidemiological studies to determine the prevalence of PBV in several groups of patient samples including; gastroenteritis outbreaks occurring between 1982-93, hospitalised patients, HIV-infected patients and sporadic cases of gastroenteritis, showed that PBV are widely distributed, but no association with disease was demonstrated.

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ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
APBV	atypical picobirnavirus
APS	ammonium persulphate
cDNA	complementary DNA
CMV	cytomegalovirus
CsCl	caesium chloride
CV	coronavirus
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ERVL	Enteric and Respiratory Virus Laboratory
GTC	guanidinium thiocynate
HIV	human immunodeficiency virus
IEM	immune electron microscopy
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBV	picobirnavirus
PCR	polymerase chain reaction
PHLS	Public Health Laboratory Service
PTA	potassium tungstic acid
RACE	rapid amplification of cDNA ends
RIA	radioimmunoassay
RPBV	rabbit picobirnavirus
RV	rotavirus
SA11	simian agent 11
SPIEM	solid phase immune electron microscopy
SRP	small round particle

SRV	small round virus
ssRNA	single-stranded RNA
TEMED	tetramethylethylenediamine
tRNA	transfer RNA
VRD	Virus Reference Division

INTRODUCTION

I) Viruses in the aetiology of infectious gastroenteritis and the 'diagnostic gap'

Acute gastroenteritis can be defined as a self limiting illness characterised by diarrhoea and vomiting. It has a brief incubation period (1 to 4 days) and the main symptoms may be accompanied by nausea, abdominal pain and cramps, low grade fever and malaise. Acute gastroenteritis occurs in both family and community outbreaks, with those affected showing a range of symptoms. In the United States of America, gastroenteritis is responsible for the hospitalisation of more than 200,000 people and for the deaths of more than 500 children each year. World-wide, gastroenteritis probably results in 3 to 5 billion episodes of diarrhoea and 5 to 10 million deaths each year, primarily in children under five years of age [Gray, 1991].

Until the 1970's, only bacteria and protozoa had been clearly demonstrated as causes of infectious gastroenteritis. It was not until the introduction of electron microscopy in the early 1970's that viruses (rotaviruses [Bishop *et al.*, 1973; Flewett *et al.*, 1973], small round structured viruses [Kapikian *et al.*, 1972; Appleton *et al.*, 1977], and adenoviruses [Flewett *et al.*, 1975]) were shown to be a cause of acute gastroenteritis.

More recently, using a broad range of diagnostic techniques, a number of viruses (Table 1.) have been implicated in the aetiology of gastroenteritis; including rotaviruses (Group A, B and C), adenoviruses (types 40 and 41), Norwalk-like viruses, caliciviruses,

and astroviruses, reviewed in [Christensen, 1989; Anon., 1990; Blacklow and Greenberg, 1991; Gray, 1991].

Other viruses have been linked to acute gastroenteritis, but their aetiological role is uncertain. They include coronaviruses [Gerna *et al.*, 1985; Mortensen *et al.*, 1985], toroviruses [Beards *et al.*, 1984; Koopmans *et al.*, 1993], parvoviruses [Paver *et al.*, 1973; Paver *et al.*, 1974; Paver and Clarke, 1976], and pestiviruses [Yolken *et al.*, 1989; Giangaspero *et al.*, 1993], (Table 2.). Coronaviruses and parvoviruses have both been found in healthy people as well as those with gastroenteritis, and their role in gastroenteritis has yet to be confirmed. Torovirus-like particles were first detected in human faeces by Beards *et al.* [1984], who also reported the preliminary characterisation of these virus-like particles [Beards *et al.*, 1986] by comparing them with the bovine torovirus (Breda virus) [Woode *et al.*, 1982] and the equine torovirus (Berne virus) [Weiss *et al.*, 1983].

Despite these advances, no aetiological agent is recognised in approximately 40% of cases of infectious gastroenteritis [Anon, 1990]. This is referred to as the 'diagnostic gap' [Flewett *et al.*, 1987]. The failure to demonstrate a known enteric pathogen in cases of gastroenteritis may be due to a number of reasons. Firstly, an inappropriate or inappropriately timed specimen may have been taken for examination. Secondly, the causative agent may be excreted in numbers below those detectable using available procedures. Thirdly, there may be other microbiological agents of gastroenteritis that are as yet unknown. The work described in this thesis is aimed at investigating whether picobirnaviruses may, at least in part, fill the 'diagnostic gap'.

Table 1. **Viruses associated with gastroenteritis in humans and their laboratory diagnosis**

Virus	Laboratory Diagnosis
Rotavirus	
Group A	Electron microscopy, PAGE, immunoassay, PCR
Group B	Electron microscopy, PAGE, immunoassay, PCR
Group C	Electron microscopy, PAGE, PCR
Enteric adenoviruses	Electron microscopy, immunoassay, PAGE, PCR
Norwalk virus	Electron microscopy, immunoassay, PCR
Norwalk-like viruses	Electron microscopy, immunoassay, PCR
Caliciviruses	Electron microscopy, immunoassay
Astroviruses	Electron microscopy, immunoassay, PCR

Table 2. **Viruses with an uncertain aetiological link to gastroenteritis in humans**

Virus	**Laboratory diagnosis**
Coronaviruses	Electron microscopy
Parvoviruses	Electron microscopy
Toroviruses	Electron microscopy, EIA
Pestiviruses	Immunoassay, PCR
Picobirnaviruses	PAGE, electron microscopy?

II) Historical perspectives and technological developments in viral gastroenteritis

i) Filterable agents of gastroenteritis

In 1929, Zahorsky proposed the descriptive name of 'winter vomiting disease' to describe an illness which was later to be termed epidemic nonbacterial gastroenteritis [Adler and Zickl, 1969]. This is the earliest clinical description of a possible Norwalk-like virus associated with gastroenteritis. However, it was not until the early 1940's, that a filterable agent (shown in the early 1970's to be a rotavirus) was isolated from the faeces of newborn infants involved in outbreaks of gastroenteritis [Light *et al.*, 1943]. The cases of diarrhoea were from six separate epidemics in the newborn in three hospitals in the USA. Stool samples from the babies were tested by culture for all known diarrhoea-causing bacterial pathogens and were negative. The stool samples were then filtered to remove bacteria and given orally to calves. These animals subsequently developed bloody and mucoid diarrhoea following an incubation period (24-48 hours) similar to that observed in the newborn infants. This was one of the first demonstrations of a putative virus causing diarrhoea in newborn infants. The agent was also passaged from calf to calf, confirming the infectious nature of the disease-causing entity.

The transmission of filterable agents associated with epidemic gastroenteritis to humans was demonstrated by Gordon *et al.* in 1947. They showed that oral administration of faecal filtrates from patients with nonbacterial gastroenteritis produced disease in human

volunteers. The filterable agent was termed the Marcy strain, after the Marcy state hospital in which an outbreak occurred. In the early 1950's the Marcy strain was again used as an inoculum for human volunteers in the Cleveland families study [Jordon *et al.*, 1953], along with the FS (family study) agent. This led to the recognition of at least two types of acute infectious nonbacterial gastroenteritis - afebrile and febrile. The afebrile illness was produced by the Marcy agent and had an incubation period of 60 hours followed by watery diarrhoea. The FS agent was responsible for the febrile illness and had an incubation period of 27 hours, followed by diarrhoea, vomiting and fever. A similar study was performed in Japan [Fukumi *et al.*, 1957], prompted by the need to investigate diarrhoeal disease which was prevalent in Japan in 1947-48. Volunteer experiments [Kojima *et al.*, 1948] were conducted that revealed the aetiological agent (Niigata strain) was filterable. It was shown in later studies that the Marcy strain [Jordon *et al.*, 1953], when given to human volunteers, produced immunity to infection with the Niigata strain [Fukumi *et al.*, 1957]. The clinical course of disease caused by both strains was virtually identical and it was concluded that the two putative viruses were closely related.

ii) The era of Electron Microscopy (EM)

a) Small round structured viruses (SRSV)

In 1968, two epidemics of winter vomiting disease were investigated in the USA. [Adler and Zickl, 1969]. One of the epidemics involved an elementary school where 50% (116 of 232) of the students and teachers developed acute gastroenteritis; the school was in Norwalk, Ohio. The illness was characterised by nausea, vomiting and abdominal cramps

and had an incubation period of 24 hours. During this outbreak, no viral or bacterial agents were isolated using the laboratory diagnosis available at the time. In 1971, a filtrate from a rectal swab specimen from an adult who presented as a secondary case of acute nonbacterial gastroenteritis in the Norwalk outbreak, induced illness in two out of three experimentally infected volunteers [Dolin *et al.*, 1972]. It was shown that the infection could be serially passaged at least two times in volunteers and still induce typical illness.

In 1972, Kapikian *et al.* used immune electron microscopy [Almedia and Waterson, 1969; Best *et al.*, 1967] and negative staining [Brenner and Horne, 1959] to visualise a 27nm particle (Norwalk virus) in material from the Norwalk outbreak. The use of immune electron microscopy (IEM) involved the aggregation virus particles in a stool filtrate from a patient with gastroenteritis with convalescent serum from experimentally infected volunteers, so that they could be more easily detected using the electron microscope. In forming aggregates of virus particles accurate measurements of particle size could be performed, even when virus was present in low concentrations in the stool specimen. This was the first demonstration of a virus associated with gastroenteritis in humans.

Other Norwalk-like agents were discovered in the 1970's and early 1980's including, the Hawaii agent [Wyatt *et al.*, 1974], the Montgomery County agent [Thornhill *et al.*, 1977], discovered in Maryland, and the Snow Mountain agent [Dolin *et al.*, 1982].

b) **The discovery of rotaviruses**

In 1973 Bishop's group in Australia described the detection of virus particles in biopsies of duodenal mucosa from children with

acute nonbacterial gastroenteritis [Bishop *et al.*, 1973]. The virus was demonstrated using thin section electron microscopy and was initially classified as a member of the orbivirus family. At about the same time reovirus-like particles measuring 75-84nm were detected by Flewett *et al.* [1973] using negative staining electron microscopy on stool samples. Negative staining has since become the method of choice for detection of rotaviruses and other faecal viruses by electron microscopy because it does not require a sample (ie. biopsy) collected by invasive procedures. These two reports described the first detection of what are now recognised as rotaviruses, a major cause of acute gastroenteritis in children.

The detection in faeces of rotaviruses in 50-60% of cases of acute diarrhoea in young children was confirmed by several workers in the early 1970's [Flewett *et al.*, 1974a; Middleton *et al.*, 1974; Flewett *et al.*, 1974b; Bishop *et al.*, 1974; Kapikian *et al.*; 1974]. Rotavirus is now recognised as the single most important cause of dehydrating diarrhoeal illness in both developed and less-developed countries, accounting for 12 to 71 percent of all episodes requiring hospitalisation in children under the age of two. In developing countries rotavirus causes roughly 125 million cases of diarrhoea annually, 18 million of them severe and leading to an estimated 900,000 deaths [Blacklow and Greenberg, 1991].

iii) Detection of other viruses associated with gastroenteritis

Two outbreaks of acute gastroenteritis in a long-stay children's ward were shown by electron microscopy to be associated with rotaviruses in one outbreak and adenoviruses in the second [Flewett *et al.*, 1975]. Tissue culture was attempted for both viruses but

neither could be grown. The rotavirus diarrhoea persisted for 2 to 15 days in the majority of cases. In the second outbreak, associated with adenoviruses, acute diarrhoea developed in six out of nineteen children and in one nurse; there was no vomiting, unlike three of six rotavirus-infected children who vomited on the first day of illness. The adenovirus-associated diarrhoea persisted for only 1-2 days. This study showed a new aetiological agent (adenovirus) could be rapidly identified using electron microscopy. Moreover, the adenoviruses could be assigned to a provisional serotype by immune electron microscopy using agglutination of virus particles with adenovirus antisera.

The extensive use of electron microscopy in the examination of faecal specimens from patients with gastroenteritis and from healthy people began to reveal further novel viruses. Madeley and Cosgrove [1975a] examined 121 cases of gastroenteritis in children and found 37% excreting rotaviruses and 29% had other round virus-like particles in their stools. These small round viruses were 28nm in size and had a distinct morphology; they had a circular outline, with a star-shaped (5 or 6 pointed) surface configuration and were often present in large numbers forming quasi-crystalline arrays. They were later to be named astroviruses [Madeley and Cosgrove, 1975b]. Appleton and Higgins [1975] described 20-30nm virus-like particles in stools from babies with mild gastroenteritis, which were shown to be astroviruses by IEM. The Marin County agent, discovered in elderly patients with diarrhoea in a convalescent hospital [Oshiro *et al.*, 1981], was also shown to be an astrovirus [Herrmann *et al.*, 1987].

Caliciviruses were known to infect other mammals before they were discovered in humans. They were first described in humans by Madeley and Cosgrove [1976] and Flewett and Davies [1976], who found them by

EM in the stools of babies with diarrhoea. The caliciviruses are about 30nm in diameter and derive their name from the 32 cup-shaped (Latin word *calyx* means cup) depressions on the surface of the virions. The surface depressions show a 'Star of David' configuration formed by six peripheral hollows surrounding a seventh central hollow. This detailed morphological description allowed caliciviruses to be clearly distinguished from the previously described astroviruses [Madeley and Cosgrove, 1975b; Madeley, 1979].

iv) **Cell Culture studies**

As a group, gastroenteritis viruses are fastidious and cannot be readily cultivated in routine cell culture.

Human caliciviruses can not be propagated in tissue culture, as demonstrated by their failure to grow in secondary rhesus monkey, primary human amnion and fetal kitten cells [Madeley and Cosgrove, 1976].

A small number of gastroenteritis viruses have, however been grown in cell cultures. This was achieved for the first time when Wyatt *et al*. [1980] adapted type 2 human rotavirus (strain Wa) to grow to high titre in primary cultures of African green monkey kidney cells after 11 passages in newborn gnotobiotic piglets. Subsequently Sato *et al*. [1981] isolated, without serial passage in animals, 3 strains of human rotavirus in cultures of MA104 cells, which are a stable cell line derived from embryonic rhesus monkey kidney. Another group in Japan [Urasawa *et al*., 1981] propagated a human rotavirus (HRV) strain in MA104 cells in a roller culture system generating large amounts of HRV and then adapted the cultivated HRV to a stationary culture system.

It was not until the early 1980's that human astroviruses were

propagated in continuous cell lines when Lee and Kurtz [1981] developed a method for their growth in primary human embryo kidney (HEK) cells. In 1990, Willcocks *et al.* succeeded in growing human astroviruses from stools by using a continuous colonic carcinoma cell line (CaCo-2). Another report described the isolation of a cytopathic small round virus in BS-C-1 cells from patients with gastroenteritis [Yamashita *et al.*, 1991]. It was concluded that this virus (Aichi strain) is either a new type of small round virus or a new serotype of astrovirus [Yamashita *et al.*, 1993].

The lack of a cell culture system has hindered the characterisation of gastroenteritis viruses and workers have had to employ other methods (e.g. EM, molecular techniques).

v) The development of Radioimmunoassays (RIA) and Enzyme-linked immunosorbent assays (ELISA)

The development of radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) for the detection of viral agents of gastroenteritis began in the 1970's. This development has been hindered by the lack of cell culture systems and these early assays utilised antigen obtained from volunteer studies. Kalica *et al.* [1977] used a microtitre solid phase radioimmunoassay to detect reovirus-like agents (rotaviruses) in human stools. The sensitivity of the RIA test was equal to that of electron microscopy (EM). The principal advantage of the RIA test was that it could be used for a large number of specimens in a single test run and could be performed in a relatively short time. A microtitre solid-phase radioimmunoassay was also developed to detect Norwalk virus antigen and antibody [Greenberg *et al.*, 1978] and Halonen *et al.* [1980] used a four-layer radioimmunoassay to detect adenovirus in stool specimens, which

proved to be a highly sensitive and specific method. Nakata *et al.* [1983] developed a microtitre solid phase radioimmunoassay to detect human calicivirus in stools, which proved to be more sensitive than EM.

The development of ELISA's took place in parallel to that of RIA's. Yolken *et al.* [1977] used an ELISA to detect human reovirus-like agents (rotaviruses) in infantile gastroenteritis. The ELISA was as sensitive as EM and RIA, but was easier to perform and when read visually did not require sophisticated technical equipment, allowing it to be employed in field studies. Herrmann *et al.* [1986] described an enzyme immunoassay (EIA) for the detection of Norwalk virus in the stools of experimentally infected volunteers. Later they used the same technique to detect the virus in samples obtained in naturally occurring outbreaks of gastroenteritis. The EIA's for Norwalk-like viruses are still not used routinely because of the lack of available antigen and antisera.

vi) Polyacrylamide gel electrophoresis (PAGE) and the detection of novel gastroenteritis viruses

In the late 1960's, Shatkin *et al.* [1968] used the technique of polyacrylamide gel electrophoresis (PAGE) [Loening, 1967] to separate the ten double-stranded RNA segments of the reovirus genome. Schnagl and Holmes [1976] subsequently used PAGE to characterise the genome of human infantile enteritis virus (rotavirus) and compared it to the genomes of other members of the family *Reoviridae*. They found that the rotavirus contained eleven segments of double-stranded RNA in its genome. Similar studies were performed by Kalica *et al.* [1976] who compared human reovirus-like agent (HRVL- rotavirus), Nebraska calf diarrhoea virus (NCDV- calf rotavirus), 'O' Agent (bovine rotavirus)

and Simian agent 11 (Simian- rotavirus) [Malherbe *et al.*, 1968]. Electron microscopy of RNA extracted from these agents revealed double-stranded RNA molecules of varying lengths. Using PAGE, the RNA segments of the human and calf viruses were divided into four size classes: class I (segments 1-4), II (segments 5 and 6), III (segments 7,8 and 9) and IV (segments 10 and 11). By contrast three size classes were seen in reovirus type 2 (Jones-strain). The other related viruses (the "O" agent and SA11 virus) differed from the human and calf viruses as well as from each other, but had the same four size classes. PAGE was thus used to separate the dsRNA genomic segments of the human and calf reovirus-like agents (rotaviruses), SA11 and 'O' Agent and differentiate between them on the basis of their electrophoretic pattern.

Using PAGE Kalica *et al.* [1978] demonstrated variation in human rotavirus genomes. They showed distinct patterns amongst eight strains of rotavirus, two from each of four successive winter epidemics of rotavirus infection (1973-1974 through 1976-1977). Strains showing different genomic patterns in PAGE became known as electropherotypes. One study in Australia by Rodger *et al.* [1981] examined the electropherotypes of rotavirus detected in specimens collected from children and babies between 1973-79. This study found 17 different electropherotypes in 116 children (aged 3 months to six years) with acute gastroenteritis. The electropherotypes showed a sequential pattern of appearance, with a limited number of electropherotypes being present at one time. By contrast, in 72 newborn babies (aged between 2 days and 2 weeks) tested, only two very similar electropherotypes were found, which did not appear in any of the older children.

In the late 1970's, an alternative PAGE technique using a discontinuous buffer system [Laemmli, 1970], was adopted by many workers [Ramig *et al.*, 1977; Rodger and Holmes, 1979; Rodger *et al.*, 1981], and replaced the technique described by Loening [1967] which used a continuous buffer system, as used by Kalica *et al.* [1976] and others. In another technical development the sensitivity of rotavirus detection by PAGE was enhanced by silver staining rather than ethidium bromide which had been used previously. Silver staining had already been used as an ultra sensitive technique to visualise polypeptides [Sammons *et al.*, 1981; Oakley *et al.*, 1980; Merril *et al.*, 1980] and other nucleic acids [Beidler *et al.*, 1982; Whitton *et al.*, 1983; Somerville and Wang, 1981] resolved by polyacrylamide gel electrophoresis. The technique was first used for rotavirus dsRNA by Herring *et al.* [1982] who were able to detect subnanogram quantities of rotavirus nucleic acid.

PAGE and silver staining were also used for the molecular characterisation of rotaviruses by Pedley *et al.* [1983], which led to the definition of three groups; A (typical), B and C (atypical). Each rotavirus group has its own unique genome profile and group antigen. Group A contained most of the rotaviruses that had been studied initially [Herring *et al.*, 1982].

The group B rotaviruses were first detected in a waterborne outbreak of rotavirus diarrhoea in China, affecting more than 12,000 adults in two mining towns [Hung *et al.*, 1984]. These viruses have since been associated with annual epidemics of diarrhoea in adults and children in China [Fang *et al.*, 1989] and more than one million cases of diarrhoea were attributed to group B rotavirus in China in 1982-83. To date no group B rotavirus infections have been detected by PAGE analysis outside China. However, antibody to group B

rotaviruses has been detected in Chinese living in the United States and other countries [Nakata *et al.*, 1987; Ushijima *et al.*, 1992]. Group C viruses have been described in humans and pigs [Bridger *et al.*, 1986; Bridger, 1987] and their importance is increasingly recognised. One of the earliest reports showing a group C genome profile by PAGE was by Rodger *et al.* [1982]. In the past few years, group C rotaviruses have been detected in human faecal specimens in many countries [Von Bonsdorff *et al.*, 1988; Ushijima *et al.*, 1989; Bothig *et al.*, 1989; Caul *et al.*, 1990; Basnec *et al.*, 1991; Maunula *et al.*, 1992]. Most group C rotaviruses have been identified in sporadic cases [Bridger *et al.*, 1986] and only a few outbreaks related to group C rotaviruses have been reported [Brown *et al.*, 1989; Matsumoto *et al.*, 1989].

vii) **Hybridisation and Polymerase Chain Reaction (PCR) Assays: New diagnostic tests**

The rapid detection of viruses has been enhanced by the use of nucleic acid hybridisation techniques. Flores *et al.* [1983], used a dot hybridisation assay to detect rotavirus in stools and other clinical samples. The assay was based on the hybridisation of labelled single-stranded RNA probes, to heat denatured rotavirus RNA from the sample immobilised on nitrocellulose membranes. The method was said to be highly specific and allowed detection of as little as 8 picograms of viral RNA (equivalent to 8 $x10^4$ particles) [Wilde *et al.*, 1990].

The development of the polymerase chain reaction (PCR) [Saiki *et al.*, 1986] which provides a technique by which the viral nucleic acid in a sample can be specifically amplified by up to 10^6-fold prior to its detection, has further enhanced the detection of viruses. A PCR

assay for the detection of rotaviruses in faeces was described by Xu *et al.* [1990]. The assay was claimed to be 100,000 times more sensitive than PAGE and 5,000 times more sensitive than hybridisation assay. Rotavirus PCR assays were also developed by Gouvea *et al.* [1990] and Wilde *et al.* [1990]. PCR was used to detect non-cultivable group B and C rotavirus [Gouvea *et al.*, 1991] and Eiden *et al.* [1991] used PCR to detect animal and human group B rotaviruses in faecal specimens.

PCR has now been used to detect a number of other viral agents of gastroenteritis. Allard *et al.* [1990] developed a PCR for the detection of adenoviruses in stool samples. The PCR was found to be a fast, sensitive and reliable method provided the amplifications were performed directly on diluted stools. They later used a two-step amplification system on NaOH-treated stool samples and were able to detect 1 copy of adenoviral DNA [Allard *et al.*, 1992]. Jiang *et al.* [1992] used a reverse transcriptase (RT) PCR to detect Norwalk virus in human stools. The RT-PCR detected virus in stool samples diluted 10^{-4} and was 100 times more sensitive than dot blot hybridisation. De-Leon *et al.* [1992] use RT-PCR and non-radioactive oligonucleotide probes to detect Norwalk virus. They used partial purification of virus from faecal material by spin column chromatography (Sephadex G-200), followed by RT-PCR.

PCR assay has also been adapted to bisegmented dsRNA viruses (birnaviruses) which are not associated with gastroenteritis. PCR was used to amplify a region of the large segment of infectious bursal disease virus [Davis and Boyle, 1990; Lee *et al.*, 1992; Wu *et al.*, 1992] and Rimstad *et al.* [1990] developed a PCR assay to detect infectious pancreatic necrosis virus (IPNV), another member of the *Birnaviridae* family.

III) Bisegmented double-stranded RNA (dsRNA) viruses

i) Bisegmented dsRNA viruses

Viruses with bisegmented dsRNA genomes infect animals, plants and fungi. The animal viruses with such genomes have been classified in the family *Birnaviridae* [Brown, 1986; Pereira, 1991; Dobos, 1991] and are characterised by a bisegmented genome, consisting of two segments of double-stranded RNA of 3300 and 3800 base pairs. The virions have a single-shell capsid and measure about 60nm in diameter with skew icosahedral symmetry and a buoyant density in caesium chloride (CsCl) of 1.32 to 1.35 g/ml. Members of this family include infectious pancreatic necrosis virus (IPNV) of fish, which is an economically important pathogen of salmonid fishes [Dobos and Roberts, 1983], and infectious bursal disease virus (IBDV) which is responsible for a highly contagious disease in chickens [Kibenge *et al.*, 1988].
A group of related viruses has been found in calves, and they have been called birna-type viruses [Vanopdenbosch and Wellemans, 1989; Vanopdenbosch and Wellemans, 1990]. This was the first report of a putative member of the virus family *Birnaviridae* in a mammalian host. The viruses were detected during an outbreak of diarrhoea in calves on a fattening farm in Belgium in 1990. In 21 out of 80 calf faecal samples tested, two dsRNA bands were detected and a virus was readily isolated in secondary fetal calf kidney cell culture. The virus initially induced a partial cytopathic effect, and when adapted to the cell line, it caused total destruction of the cell monolayer. The virus particle identified in faeces and cell culture supernatant by electron microscopy was an icosahedral, non-enveloped virion, with a

diameter of approximately 40nm. The genome profiles of these birna-type viruses [Vanopdenbosch and Wellemans, 1990] show patterns of genomic segments similar to that of birnaviruses [Dobos and Roberts, 1983].

Cryptoviruses are bisegmented dsRNA viruses that infect plants [Boccardo *et al.*, 1987]. They are divided into two subgroups represented by white clover cryptic virus 1 (WCCV1) and WCCV2 respectively [Pereira, 1991]. The two genomic segments of these viruses are contained in isometric capsids either 30nm in diameter with no surface structure (WCCV1), or 38nm in diameter with distinct capsomers (WCCV2).

Mycoviruses are viruses that infect fungi. Bisegmented dsRNA mycoviruses [Buck *et al.*, 1984] have been classified in the family *Partitiviridae* (dsRNA mycoviruses with divided genomes) and in the bipartite dsRNA mycovirus subgroup [Buck and Ghabrial, 1991].

ii) **Mammalian and avian picobirnaviruses**

The name picobirnavirus was suggested for a new group of viruses described by Pereira *et al.* [1988b] and is derived from the prefix pico- meaning small (virion size) and a combination of the letters in the phrase bisegmented dsRNA virus, as in birnaviruses [Pereira, 1991]. The two genomic segments of picobirnavirus dsRNA are approximately 1500 and 2500 base pairs in length. The virions are small round particles with no distinct surface structure, measure approximately 35nm in diameter and have a buoyant density in CsCl of 1.38 to 1.4 g/ml.

The first picobirnavirus (and first bisegmented dsRNA virus) to be

detected in a mammalian host was isolated from the intestinal contents of a wild rat (*Oryzomys nigripes*) [Pereira *et al.*, 1988a]. The picobirnaviruses were detected by chance during rotavirus screening of the wild rat population in the Vale da Ribeira region of the state of San Paulo, Brazil. The animals were trapped and killed. Blood, whole intestinal contents and faeces were collected and stored until analysed. PAGE analysis of nucleic acid extracted from faecal samples led to the observation by Pereira *et al.* [1988b] of two bands of nucleic acid that appeared to be equimolar by staining intensity and were later shown to be representative of the genome of this novel group of viruses.

A picobirnavirus (PBV) was also described in pig faeces by Gatti *et al.* [1989] in Brazil. They examined faecal samples from 912 pigs and found PBV in 106 pigs (11.6%). Picobirnaviruses alone or together with rotaviruses were detected in 49 (15.3%) of 321 animals with diarrhoea and in 57 (9.6%) of 591 animals without diarrhoea. Ludert *et al.* [1991], identified picobirnaviruses in pigs in the State of Carabobo in Venezuela. They examined 244 faecal samples and found PBVs in 10-12% of both diarrhoeic and normal animals, with the agent being more prevalent (17%) in 15 to 35 day old piglets than in other age groups. A picobirnavirus has also been detected in pig faeces in the United Kingdom by Chasey, [1990].

Pereira *et al.* [1989] described a picobirnavirus in guinea pig faeces and intestinal contents. They examined 102 samples and found one picobirnavirus. The virus was inoculated into several cell lines including guinea pig embryo cells, human diploid fibroblasts (MRC5), primary or secondary monkey kidney and in HEp2 human cells. The characteristic two bands were detected in cell culture material from the first but not subsequent passages. Inoculation of permanent cell

lines, including hamster (BHK-21), simian (VERO, MA104) and porcine (IBRS-2) gave negative results. The presence of viral nucleic acid in some but not other cell types inoculated and maintained under the same conditions indicated that the PBV nucleic acid detected was not derived from residual virus in the inoculum. It was therefore suggested that in certain cell cultures the virus underwent a cycle of abortive replication, resulting in the synthesis of viral RNA.

An avian bisegmented dsRNA virus (picobirnavirus) was described in chicken faeces [Alfieri *et al.*, 1989]. The virus was detected in 17 (14%) of 120 faecal samples collected from 25 to 30 day old chickens (with gastroenteritis) in Parana and Minas Gerais States, Brazil. This virus was tentatively named picodiplornavirus, but is now regarded as a picobirnavirus. Another group of workers in Brazil detected picobirnaviruses in the large intestinal contents of 44 (17.5%) of 257 chickens obtained from a slaughter house in Bom Jardim, State of Rio de Janerio, Brazil [Leite *et al.*, 1990] and similar viruses in chickens were also described by others in Brazil [Monteiro *et al.*, 1991].

iii) Picobirnaviruses in humans

The detection of picobirnaviruses in humans was first reported by Pereira *et al.* [1988a]. They found the two characteristic genomic bands in 14 of 3134 faecal samples (0.45% overall detection rate) from children with acute gastroenteritis. A similar study in Venezuela by Ludert and Liprandi [1993] revealed the presence of picobirnaviruses in 1 (0.5%) of 213 hospitalised children with diarrhoea. In a recent study on enteric viruses and diarrhoea in HIV-infected patients in the USA, Grohman *et al.* [1993] reported the detection of picobirnaviruses by PAGE. They showed that

picobirnaviruses were found significantly more often in faecal samples from patients with diarrhoea (9%) than in samples from patients without diarrhoea (2%). This implied that these faecal viruses may be a cause of diarrhoea in HIV-infected patients. Work now described in this thesis represents the first report of picobirnaviruses in humans in the United Kingdom [Brown *et al.*, 1990; Gallimore *et al.*, 1991, Gallimore *et al.*, 1995a, Gallimore *et al.*, 1995c], and a bisegmented dsRNA virus associated with *Cryptosporidium* positive stools in humans [Gallimore *et al.*,1995b]. Also presented here for the first time is the detection and characterisation of picobirnaviruses in rabbits [Gallimore *et al.*, 1993].

The detection and characterisation of picobirnaviruses in humans and rabbits is now described.

AIMS and OBJECTIVES

The aim of this work was to investigate the role of picobirnaviruses in the pathogenesis of gastroenteritis in humans and determine if picobirnaviruses are one of the viral agents responsible for some of the 40% of undiagnosed cases of gastroenteritis in humans. Firstly, the work describes the preliminary observations of two equimolar bands in human faecal samples and characterisation of these bands and associated virus particles to confirm the picobirnavirus criteria. During the course of this work methods for the detection of picobirnaviruses in faecal samples were be optimised. A number of epidemiological studies were undertaken to determine the incidence of PBV in outbreaks of gastroenteritis, hospitalised patients with and without gastroenteritis, HIV-infected patients, and sporadic cases of gastroenteritis. The study of these novel agents would also be greatly aided by an *in vitro* tissue culture system or an *in vivo* animal model for propagation of virus; both of these were investigated.

METHODS

MM1) Preparation of faecal extracts from humans and animals

Faecal samples from humans were prepared as 10% faecal extracts in Tris/Ca buffer (see appendix). The preparation was vortexed for 10 sec and then centrifuged at 800x g (1850 rpm, MSE 3000 centrifuge) for 5 min at 5°C [Herring et al., 1982]. The supernatant was stored at 4°C until required. Faecal samples from rabbits and other animals were generally in the form of dry or semi-dry pellets. This material was treated essentially the same as human faeces, except that the faecal extract was placed on a rocker for 30 min to several hours after the vortexing, to mix the sample thoroughly.

MM2) Nucleic acid extraction from faecal samples for PAGE analysis

a) Phenol/chloroform extraction and ethanol precipitation

A 450ul 10% faecal extract was added to 50ul of 1M sodium acetate, pH 5, containing 1% sodium dodecyl sulphate (SDS). The mixture was vortexed for 10 sec and an equal volume of 70% phenol/5% water/25% chloroform (Applied Biosystems) was added. This was vortexed and then incubated at 56°C for 15 min. The sample was centrifuged at high speed, 10,000x g (13,000 rpm, MSE-microcentrifuge) for 2.5 min. The upper aqueous phase was removed and added to a clean microcentrifuge tube (the lower phase containing the phenol was discarded). To this was added 50ul (1/10 vol) of 3M sodium acetate, pH 5.5, and 1ml (2 vol) of cold ethanol. This mixture was placed at -70°C for 1h or -20°C for 2h or overnight. Following this ethanol precipitation, the nucleic acid was pelleted by high speed centrifugation (MSE-

microcentrifuge) for 15 min. The supernatant was removed and the pellet washed with 500ul of 70% ethanol and centrifuged for 5 min. The supernatant was removed and the pellet dried under vacuum. The microcentrifuge tube containing the dried pellet of nucleic acid was stored at 4°C or resuspended in 50ul of TE (see appendix) until use.

b) Guanidinium thiocynate (GTC)/silica extraction of nucleic acid - The 'Boom' method.

This method was described by Boom *et al.* [1990] and was modified for use for extraction of nucleic acid from faeces. The method uses guanidinium thiocynate to disrupt the protein coat of viruses and liberate nucleic acid. The nucleic acid is bound to silica, which is washed and the nucleic acid is then eluted from the silica. To 500ul of a 10% faecal extract was added 1ml of L6 buffer (see appendix) and 10ul of size fractionated silica (see appendix). This was vortexed for 10 sec and incubated at room temperature for 30 min. The mixture was pelleted by high speed centrifugation (MSE-microcentrifuge) for 15 sec and the supernatant was removed. The pellet was washed twice with 1ml of L2 buffer (see appendix), twice with 1ml of 70% ethanol and once with 1ml of acetone. The acetone was removed from the pellet and the tube was incubated at 56°C in a dry heating block for 15 min (with the microcentrifuge tube lid open to allow the remaining acetone to evaporate). The nucleic acid was eluted by adding 50ul of TE buffer and incubating at 56°C for 15 min (with the microcentrifuge tube closed). The mixture was pelleted by high speed centrifugation (MSE-microcentrifuge) for 2 min and the supernatant containing the nucleic acid was collected and stored at 4°C until required.

c) **RNAzol and the 'Boom' method - an alternative extraction procedure**

The procedure combines a preliminary step using RNAzol (Biogenesis) which is a mixture of phenol and guanidinium thiocynate, with the 'Boom' method described earlier. Studies with rotaviruses showed that using RNAzol as a preliminary step gave cleaner preparations than 'Boom' alone (data not shown).

To 500ul of a 10% faecal extract was added 500ul of RNAzol (Biogenesis). This was vortexed for 10 sec and 100ul of chloroform was added. Again, the mixture was vortexed for 10 sec, and incubated on ice for 5 min. At this stage the addition of chloroform causes the mixture to separate into two phases, one containing the phenol and chloroform (lower phase), and the other containing the guanidinium thiocynate and the liberated nucleic acid in solution (upper phase). The mixture was centrifuged at high speed (MSE-microcentrifuge) for 15 min. The upper phase was added to a clean tube, leaving the lower phase and the interface which consists of the protein debris. To the upper phase was added 10ul of silica and 750ul of L2 buffer, the mixture was incubated at room temperature for 30 min. The mixture was then pelleted by high speed centrifugation (MSE-microcentrifuge). This was followed by one other washing step with L2 and the remaining steps of the 'Boom' method.

MM3) Polyacrylamide gel electrophoresis (PAGE).

The PAGE technique used was originally described by Laemmli, [1970], but here a non-denaturing system was used for the separation of nucleic acids as described by other workers [Sethi *et al.*, 1988; Noel *et al.*, 1991]. A PAGE gel was prepared in a Hoeffer vertical

electrophoresis apparatus. The gel was cast between two glass plates (16 x 16cm), separated by two 1.5mm spacers. The PAGE gel consists of two parts; a resolving gel and a stacking gel. To make the first part of the gel, the resolving gel, a 10% acrylamide resolving gel mixture was prepared by adding 15.8ml water, 10ml acrylamide/bis solution 30:0.8 (v/v) (Severn Biotech), 3.75ml resolving buffer (see appendix) and 15ul TEMED (Sigma). Polymerisation of the gel was activated by adding 200ul 10% (w/v) ammonium persulphate (APS) and the gel was poured immediately to a mark which was approximately 3cm below the bottom of the sample comb; this allows room for the stacking gel. The resolving gel was overlaid with butan-2-ol saturated with water to prevent a meniscus forming at the sides of the gel. The gel set in 30 min and the butan-2-ol/water was poured off and the top of the gel was washed with water to remove any traces of butanol. The stacking gel was prepared by adding 6.8ml water, 1.7ml acrylamide/bis solution, 1.25ml stacking gel buffer (see appendix), 5ul TEMED. Polymerisation of the stacking gel was activated by adding 100ul 10% APS and poured on top of the resolving gel. The sample comb was placed between the glass plates in the stacking gel to form the wells. The stacking gel set in approximately 15 min, the comb was then removed, and the wells were filled with electrophoresis tank buffer (see appendix).

Nucleic acid samples for PAGE analysis were mixed with an equal volume of sample buffer (see appendix) and loaded into the wells formed by the comb. The samples were electrophoresed for 4h at 40mA per gel or 18mA per gel overnight (16h) at $4^{o}C$.

Rotavirus SA11 control and molecular weight marker for PAGE

This was supplied as a 1ml aliquot of freeze-dried SA11 (rotavirus simian agent 11) from Dr G. Beards (Regional Virus Laboratory, Heartlands Hospital, Birmingham). Its preparation and culture is described in Materials and Methods 11 (MM11). An aliquot of SA11 (100ul to 250ul) in tissue culture fluid was extracted with each batch of faecal samples and used as the extraction control. SA11 was also used as a molecular weight marker as the size of all eleven segments has been determined [Mitchell and Both, 1990].

MM4) Silver Staining of PAGE gels

The method described here is a modification of that used by Herring *et al.* [1982]. The gel was removed from the glass plates and placed in a clear plastic box on an orbital shaker and washed for 30 min in a solution of 40% methylated spirits (meths), 5% acetic acid. This solution was replaced by 10% meths, 0.5% acetic acid and the gel washed for a further 30 min. This washing solution was drained off and 200ml of silver stain (see appendix) was added to the gel for 30 min, shaking gently. The stain was removed and the gel was washed twice for 2 min with water to remove any trace of excess silver stain on the surface of the gel. The staining pattern of the nucleic acid bands on the gel was developed by adding 250ml developer (see appendix). The gel was first washed with 50ml of developer for 10 sec, which was then removed and replaced with the remaining 200ml of developer. The gel was placed back on the orbital shaker until the nucleic acid bands were visible. The gel was fixed by removing the developer and replacing it with 5% acetic acid. The stained gel was stored in 10% meths, 0.5% acetic acid. The gels were photographed

using a standard 35mm camera by the Medical Illustration Department at CPHL.

MM5) Nuclease Digestions

Nuclease digestions were performed as previously described [Pereira *et al.*, 1988b]. Briefly, pancreatic RNase A (Sigma), RNase T1 (Life Technologies) and RQ1 DNase (Promega), were used at final concentrations of 40ug/ml, 100 units (U)/ml and 100 U/ml respectively. The control substrates were rotavirus SA11 (dsRNA), yeast tRNA (ssRNA- type V, Sigma) and ØX174 DNA/Hae III (dsDNA- Life Technologies), were used at final concentrations of 1ug/ml, 100 U/ml, and 40ug/ml respectively. An alternative dsDNA substrate used was 1Kb marker (Life Technologies) at a final concentration of 20ug/ml. Test samples were digested at 37^{o}C for 1 h and the reaction mixture was added to an equal volume of sample buffer and incubated at 56^{o}C for 15 min. The samples were then examined by PAGE.

MM6) Purification of picobirnaviruses from human faeces and determination of buoyant density in caesium chloride (CsCl)

The starting material for the purification of PBV from human samples were either solid faeces or liquid faeces. This was added to Tris/Ca buffer (see appendix) to make a 10% faecal extract and 5ml was the starting volume. The mixture was vortexed and centrifuged at 1850 rpm (MSE 3000 centrifuge) for 5 min at 5^{o}C to clarify the mixture. The supernatant was transferred to a glass universal and an equal volume of trichlorotrifluoroethane (Arklone- Agar Scientific

Ltd) was added. The mixture was vortexed for 1 min and then centrifuged as before. The supernatant (4ml) was then layered on top of 1ml of 45% sucrose and centrifuged for 2 h at 5^{o}C at 150,000x g (40,000 rpm, SW55 Ti rotor, Beckman L8-80 M ultracentrifuge). The supernatant was discarded and the pellet was resuspended in 350ul of Tris/Ca buffer. A 300ul aliquot was layered on top of 4.7ml of 30% CsCl solution (60% CsCl v/v : Tris/Ca buffer [50:50]) and centrifuged for 17.5 h at 5^{o}C at 125,000x g (35,000 rpm, SW55 Ti rotor, Beckman L8-80M ultracentrifuge). The CsCl gradient was fractionated by collecting sixteen 300ul fractions from the top. A 10ul aliquot was used to determine the refractive index of each fraction using a refractometer (Bellingham & Stanley Ltd.) and its corresponding buoyant density was calculated from standard tables (see A4- Table 27.). A 40ul aliquot of each fraction was then used for electron microscopy (MM7) and 250ul was used for PAGE. The samples for PAGE analysis were diluted with an equal volume of Tris/Ca buffer and extracted using the 'Boom' method (see MM2) and electrophoresed on a standard PAGE gel (MM3) and the gel was silver stained (MM4).

MM7) Electron Microscopy (EM) of human faeces purified by CsCl gradient centrifugation

CsCl fractions were stored at 4°C for a few hours until required for examination by negative staining electron microscopy. A 10ul aliquot of each fraction was placed on a microscope slide coated with 3ml 0.9% agarose, so that it formed a drop on the surface of the agarose. A formvar-carbon coated grid was then placed face down on top of the drop. The grids were incubated at room temperature for 30 min, by which time the drop had diffused into the agarose and the grid was lying on the surface of the agarose. This procedure was used to remove the CsCl from the the fraction sample, because the presence of high concentrations of salts such as CsCl obscures the detection of viruses by electron microscopy. The grid was then inverted and 10ul of 3% phosphotungstic acid (PTA), pH 6.5, was added to the grid. The grid was then blotted dry by applying a small piece of filter paper to one edge from the side and stored in a grid tray in a dry box until examined by EM using a Philips 420 transmission electron microscope.

MM8) Direct electron microscopy of human faeces

Two sample preparation procedures were used as two separate laboratories were performing these methods. The direct EM was undertaken by David Lewis at Leeds PHL, and Dr Hazel Appleton in VRD at CPHL.

Leeds PHL method - (D.Lewis)

A 25% faecal extract was made in phosphate buffered saline (PBS) and centrifuged at 2000x g for 15 minutes. A 30ul aliquot of the

supernatant was taken and a carbon coated, grid with formvar support film was placed on top for 1.5 hours at room temperature in a moist chamber. The grid was lifted off and blotted dry from the edge and stained with 2% phosphotungstic acid (PTA) pH 6.6. The grid was viewed in a JEOL 1200 EX transmission electron microscope and the microscope was calibrated using catalase crystals.

VRD method - (H.Appleton)

A 10% faecal extract was made in Medium 199 with Earles salts (Imperial Laboratories) and centrifuged at 750x g (1850 rpm, Mistral 3000) for 10 minutes. A 1ml aliquot of the supernatant was centrifuged at 31,000x g (20,000 rpm, Sorvall RC3B) for 1 hour and the pellet was resuspended in distilled water. A drop of the faecal suspension was mixed with an equal volume of a 1:1 mixture of 3% PTA pH 6.5 and 0.05% bovine serum albumin. A formvar-carbon coated EM grid was placed on a drop of this mixture and left at room temperature for 2 minutes and then blotted dry. The grid was examined in a Philips 420 transmission electron microscope and the microscope was calibrated using catalase crystals.

The diameter of the PBV-like particles were measured in millimeters from the negative using a standard equation.

Size in nm = diameter of particle (mm) x 10^6 / magnification.

For example,

PBV particle size = 1.85 x 10^6 / 57,000 = 32nm

MM9) **Animal Studies**

a) **Study 1. Inoculation of various small animals with two strains of human picobirnaviruses.**

The animal project licence (no. PPL 70/01628) was held by Dr David Brown and the animal procedures were performed by David Conway (Biological Services Division, CPHL) and Dr David Lees (VRD).

Inoculation

Two human faecal samples 39177/89 and 21975/89 containing putative picobirnaviruses were used as the virus inoculum. A 1% and 10% faecal extract was prepared in minimal essential media (MEM- Gibco-BRL), with gentamycin. A volume of 25ml was prepared for each sample and the control MEM with no faecal sample. The faecal extract was sequentially filtered through a 0.8um, 0.45um and a 0.2um syringe filter. An aliquot of the post 0.2um filtrate was examined by PAGE to see if the virus bands were still present. A 100ul aliquot was also inoculated into 20ml of broth culture to check for bacterial contamination. The animals in this study included six each of, rabbits (*Oryctolagus cuniculus*, strain New Zealand White), cavies (*Cavia porcellus*, strain Dunkin-Hartley), rats (*Rattus norvegicus*, strain Spargue-Dawley), hamsters (*Mesocricetus auratus*, strain Syrian) and mice (*Mus domesticus domesticus*, strain Schofield). The rabbits were housed one per cage, and the guinea pigs and rats were housed two per cage in mesh bottomed cages. The hamsters and mice were housed two per cage in metabolism cages. Infected animals were housed separately to uninfected animals. Inoculation of the animals was by the oral route using oral dosing catheters. The animals were anaethetised before inoculation. The volumes of inoculum were adjusted to give a constant volume/average body weight ratio [see Table 3.].

Table 4. **Cage distribution of study 1 animals**

Animal		PBV strain	Cage
rabbit	1 & 2	control	1 & 2
rabbit	3 & 4	39177/89	3 & 4
rabbit	5 & 6	21975/89	5 & 6
rat	1 & 2	21975/89	7
rat	3 & 4	31977/89	8
rat	5 & 6	control	9
cavie	1 & 2	39177/89	10
cavie	3 & 4	21975/89	11
cavie	5 & 6	control	12
hamster	1 & 2	39177/89	13
hamster	3 & 4	21975/89	14
hamster	5 & 6	control	15
mouse	1 & 2	39177/89	16
mouse	3 & 4	21975/89	17
mouse	5 & 6	control	18

The animals were sacrificed after 1 month and the maximum volume of blood was taken from each animal by heart puncture.

b) Study 2. **Inoculation of adult rabbits with a putative rabbit picobirnavirus and a human picobirnavirus.**

The rabbits in this study were inoculated using the same procedure as in study 1. However the faecal samples used varied. Rabbits 13 and 14 were inoculated with the control, rabbits 15, 16 and 17 with human PBV (21975/89) and rabbits 18, 19 and 20 with rabbit PBV (R5-9). Pre-inoculation sera were collected from all rabbits. Terminal sera were collected from rabbits 13, 14, 15 and 16, by heart puncture. Faecal samples were collected for 19 days for each rabbit.

c) Study 3. **Inoculation of newly weaned rabbits with a putative rabbit picobirnavirus (R5-9).**

The young rabbits in this study were inoculated according to the procedure in study 1. The inoculum used for all 10 rabbits was the faecal sample containing rabbit picobirnavirus (R5-9), and there were no control rabbits. Faecal samples were initially collected from day 0 (pre-inoculation) to day 14. The rabbits were housed in separate cages but were housed two to a room. Rabbit 1 & 2 were in room A, rabbit 3 & 4 in room B, rabbit 5 & 6 in room C, rabbit 7 & 11 in room D, and rabbit 9 & 10 in room E. No pre-inoculation sera were taken from the young rabbits used in this study. Acute sera were taken at day 4 post inoculation and the convalescent sera were taken at day 21 after inoculation.

d) Study 4. Re-inoculation of some rabbits used in study 2 and study 3 with a putative rabbit picobirnavirus (R5-9).

The inoculation protocol used in this study was the same as previously used for the other studies. The rabbits used for re-inoculation were rabbit 17 and rabbit 20 from study 2, and rabbit 1, 2, 4, 5, 6, 10 and 11 from study 3. The inoculum used in this study was the faecal sample R5-9. Faecal samples were collected from day 0 (pre-inoculation) to day 10 in most of the rabbits. Termination sera were collected from all rabbits at the end of the study.

MM10) Immune electron microscopy (IEM) of rabbit picobirnaviruses

Immunoglobulin G (IgG) was purified from 200 ul of rabbit serum (rabbit 5 study 1 or rabbit 6, 10 or 11 from study 3) by affinity chromatography on DEAE Affi-gel blue (Bio-rad) according to the manufacturer's recommended method. The peak 4ml fraction of IgG was collected for each serum. The IEM was performed by D.Lewis at Leeds PHL.

a) Solid phase immune electron microscopy (SPIEM)

For the SPIEM method [Lewis *et al.*, 1988], carbon coated grids with formvar support films were floated for 30 min at 37^{o}C in a moist chamber on 25 ul drops of protein A (Sigma) diluted to a protein concentration of 50 ug/ml in phosphate buffered saline (PBS). The grids were coated with an appropriate dilution (1:10 to 1:30) of purified rabbit IgG. The grids were washed on two drops of PBS before being floated on the faecal extracts for 90 min at 37^{o}C. The grids were washed as before and then stained with 1.5% potassium phosphotungstate.

b) **Immune clumping/SPIEM**

For the immune clumping method, 25 ul of rabbit IgG and 25 ul of faecal extract were mixed and incubated at 37^{o}C in a moist chamber for 90 min. IgA coated grids prepared as in the SPIEM method above were then placed on the drops of faecal extract/IgG mixture and incubated for a further 90 min. The grids were washed and stained.

Grids were viewed in a JEOL 1200 EX transmission electron microscope and particles were at 100,000 times magnification and calibrated using catalase crystals.

The mean measurements of 15 virus diameters was calculated.

MM11) **Inoculation of various cell cultures with picobirnaviruses**

Propagation of picobirnaviruses by an *in vitro* method with several cell lines was investigated.

The general procedure for inoculation for all the cell lines used was based on a rotavirus (SA11) inoculation protocol.

a) **The growth of SA11 in MA104 (African green monkey kidney) cells**

For the propagation of SA11, MA104 cells were grown in EMEM medium (see appendix) in 75cm^{2} disposable cell culture flasks until confluent. The virus was diluted from stock virus (reconstituted freeze-dried SA11 in 1ml of distilled water) 1/50 in EMEM medium (without foetal calf serum) with 10ug/ml trypsin. The EMEM medium was poured off the cell sheet and the cells were washed with phosphate buffered saline (PBS). The cell sheet was inoculated with 1ml diluted SA11 and the flask was incubated at 37^{o}C for 30 min, and the inoculum

was then poured off. EMEM medium (without foetal calf serum) with 1ug/ml trypsin was added to the cell sheet and the flask was incubated at 37°C for 5 to 7 days depending on cytopathic effect (CPE). Harvesting of the virus was by freeze/thawing the cells.

b) **Propagation of picobirnaviruses in cell culture**

i) **Human embryo kidney (HEK) cells**

These cells were grown in two 75cm^2 cell culture flasks, one flask was inoculated with a picobirnavirus from human faeces (21975/89) and the other flask with R5-9 from rabbit faeces. A 10% faecal extract was treated with 10ug/ml trypsin and absorbed onto the cell sheet for 30 min at 37°C. The supernatant was poured off and replaced with HEK maintenance medium (without FCS) with 1ug/ml trypsin. Both flasks were observed for 7 days for any sign of CPE. The tissue culture fluid was collected and nucleic acid was extracted and analysed by PAGE.

ii) **Human embryonic lung (MRC5) cells**

This cell line was inoculated with R5-9 and 21975/89 as described for HEK cells. The MRC5 maintenance medium that was added had 10% foetal calf serum added and no trypsin. The tissue culture fluid was analysed as previously described.

iii) **Rabbit kidney cells**

These cells were purchased as primary cell line in cell culture tubes (Biowhittker), already confluent. EMEM medium was used to maintain the cell line. The picobirnavirus strains used to inoculate

these cells were from four rabbits. R5-9 from animal study 1 and R6-9, R7-1 and R10-12 from animal study 3. The control was faecal extract medium. Each of the samples were prepared as 10% faecal extracts. Two slightly different inoculation procedures were used, but both were based on the rotavirus SA11 protocol.

Inoculation procedure- A

To 100ul of faecal extract was added 10ug/ml trypsin and this was incubated at 37^{o}C for 30 min. The medium was poured from the cell sheet and the inoculum was added, the cells were then incubated at 37^{o}C for 1h. The supernatant was poured off and 1ml of EMEM (with 10% FCS) was added.

Inoculation procedure-B

Here the procedure was essentially the same, however EMEM (no FCS) with 1ug/ml trypsin was added instead of that described for procedure- A.

All tubes were incubated at 37^{o}C for 5 days and observed for any CPE. After 5 days the cells were freeze/thawed, and 100ul of TCF from each tube was passed into the second set of primary rabbit kidney cells in tubes. The TCF used as the inoculum was treated the same as the faecal extract samples used in the first inoculation. The procedure was then repeated for a second passage. All TCF samples were stored at -20^{o}C and analysed by PAGE for the dsRNA genome of picobirnaviruses.

MM12) Purification and concentration of Cryptosporidium oocysts

This procedure was used to attempt to co-purify *Cryptosporidium* oocysts and atypical picobirnaviruses to demonstrate if the atypical PBV infected the *Cryptosporidium* oocysts. The method is designed to concentrate *Cryptosporidium* oocysts and is a modified formol ether method [Casemore, 1991].

An emulsion was prepared by adding 0.5g faeces to 3ml 10% formalin in water and vortexing. To this was added 3ml ether and the mixture was mixed vigorously for 30 to 40 sec. The mixture was made up to 15ml with 10% formalin, remixed and centrifuged at 450 x g in a Sorvall RT6000 for 2 min. The fluid between the ether and the deposit was removed and placed in a new tube. To this was added 10% formalin up to 15ml and it was then centrifuged at 100 x g for 5 to 10 min. The supernatant was discarded. The *Cryptosporidium* oocysts were concentrated in the pellet which was resuspended in 250ul PBS. A 200ul fraction of the sample was used for the 'Boom' dsRNA extraction procedure previously described and the nucleic acid was then analysed by PAGE.

RESULTS

SECTION 1 Human picobirnavirus characterisation

The results are divided into four sections as follows: Section 1, covers the characterisation of the picobirnavirus genomic segments detected in human faeces, the characterisation of picobirnavirus-like particles and the optimisation of detection methods.

R1) Initial detection of picobirnaviruses in human faeces by PAGE analysis.

R2) Sizing of picobirnavirus genomic segments.

R3) Characterisation of PBV nucleic acid by nuclease digestions.

R4) Determination of buoyant density in CsCl of picobirnavirus particles from human faeces by PAGE analysis.

R5) Electron microscopy of picobirnavirus-like particles in human faeces.

R6) Optimisation of various parameters of the 'Boom' method for extraction of dsRNA from faeces.

R1) Initial detection of picobirnaviruses in human faeces by PAGE analysis

Picobirnaviruses were initially detected in human faeces during the screening of samples from outbreaks of gastroenteritis for group C rotaviruses. RNA prepared from faecal extracts by phenol/chloroform extraction were analysed by PAGE, followed by silver staining as

described in MM1-MM4. The screening did not reveal any group C rotaviruses, apart from one particular outbreak [Brown *et al.*, 1989]. However, in a small percentage (approximately 5%) of the samples two equimolar bands were present which resembled the bisegmented genome of picobirnaviruses. These genome profiles are demonstrated in Figures 1. to 3. In one sample (Figure 3., lane 2), two bands are shown for each segment. It is possible that this was due to a mixed infection with two very similar picobirnavirus strains with slightly different genome profiles, although it may be due to an artefact of silver staining.

The genome profiles demonstrated in Figures 1 to 3 were from faecal samples collected between 1982 and 1989 and stored at -40^{o}C. These results went on to form part of an epidemiological study of picobirnaviruses in human faeces which is described later (R7).

An additional picobirnavirus strain (21975/89) was detected in the faeces of an adult male who had mild diarrhoea for only 1 day in the early part of the summer of 1989. A faecal sample had been submitted to the ERVL for virological investigation. The genome profile of PBV strain 21975/89 is demonstrated in Figure 4.

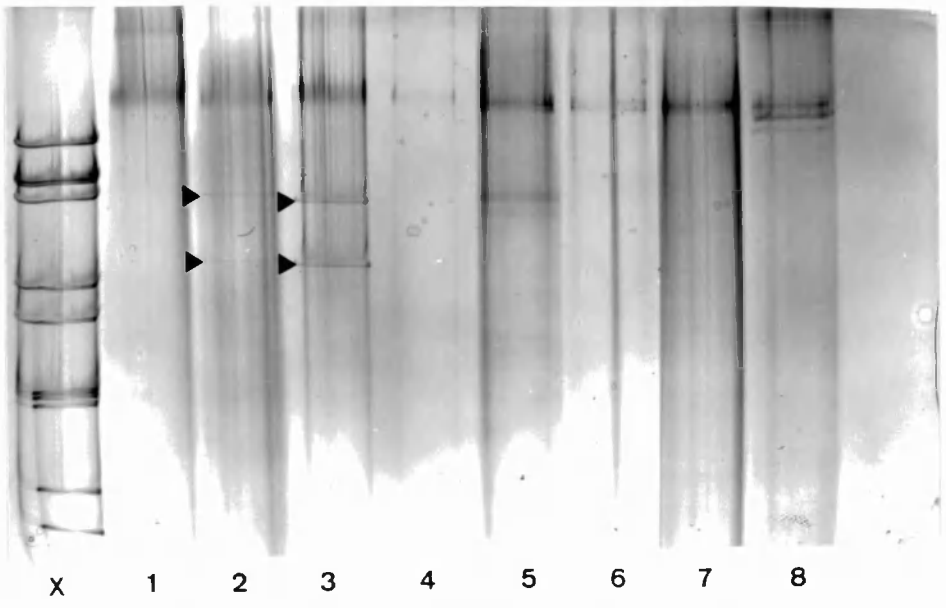


Figure 1. PAGE analysis of human faecal samples
Lane 2 and 3 demonstrate a picobirnavirus-like genomic profile. Lane X is a group A rotavirus control

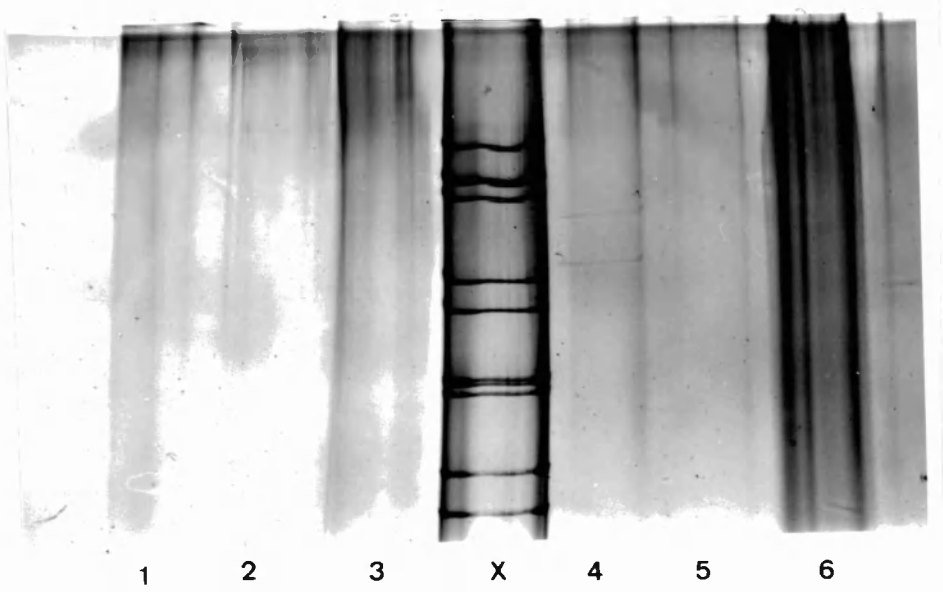


Figure 2. PAGE analysis of human faecal samples
Lane 4 demonstrates a picobirnavirus-like genomic profile. Lane X is a group A rotavirus control

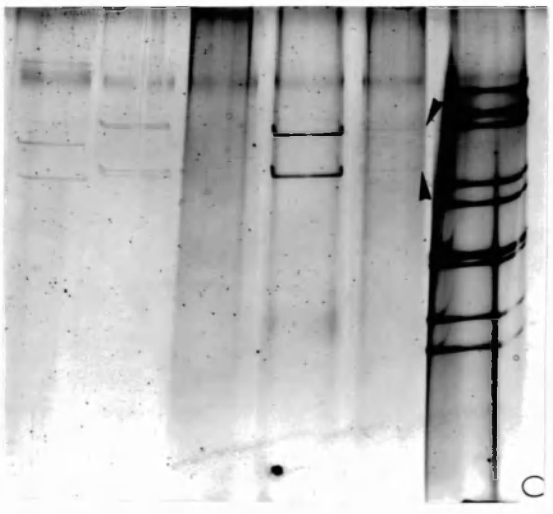


Figure 3. **PAGE analysis of human faecal samples**

Lane 1, 2, 4 and 5 demonstrate several picobirnavirus-like genomic profiles. Lane X is a group A rotavirus control

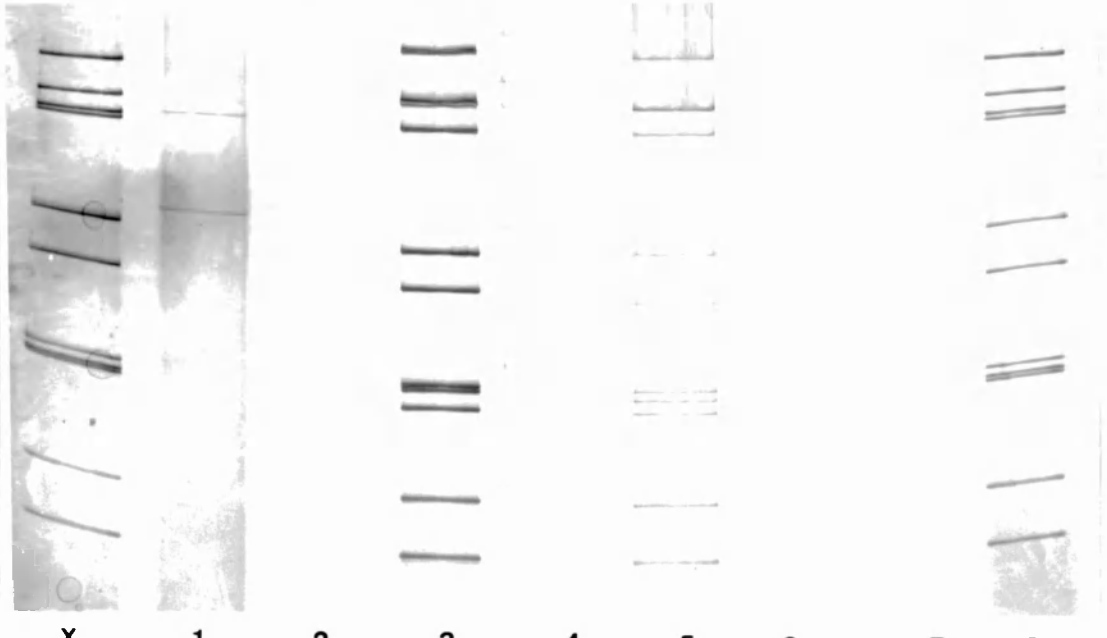
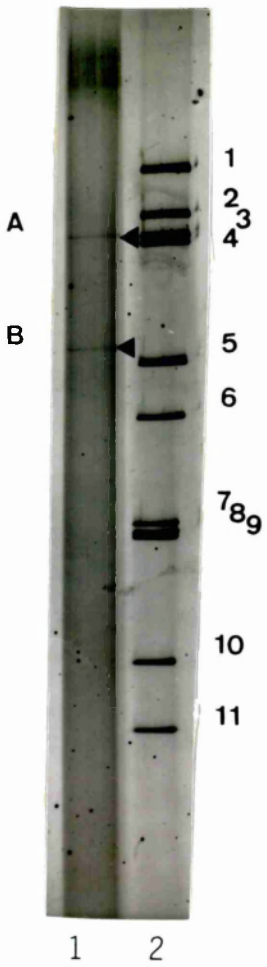


Figure 4. **Demonstration of picobirnavirus strain 21975/89 by PAGE analysis**

Lane 1. PBV strain 21975/89. Lanes 3, 4, 5 and 8 contain group A rotaviruses (part of an ongoing screening study at the time). Lane X is SA11 control, which was used as a extraction control and molecular weight marker (see MM3)

R2) **Sizing of picobirnavirus genomic segments**

The sizes of the two genomic segments of picobirnavirus strain 21975/89 were estimated by comparing the electrophoretic mobility of the two PBV segments with that of simian rotavirus SA11 for which all eleven segments have been fully sequenced [Mitchell and Both, 1990]. The sizes of segments were calculated from a plot of the migration distance of SA11 genomic segments against Log_{10} of the number of nucleotide pairs or base pairs (bp) (Tables 5. and Figure 6.).



SA11 Segments	Genome size (bp)
1	3303
2	2690
3	2591
4	2362
5	1611
6	1356
7	1104
8	1059
9	1062
10	751
11	667

Figure 5. **Sizing of picobirnavirus genomic segments using electrophoretic migration in a PAGE gel**

Lane 1. PBV 21975/89 segments A and B and lane 2. simian rotavirus SA11 segments 1 to 11

Table 5. Migration distance and sizes of SA11 genomic segments used to plot the calibration curve and PBV 21975/89 genomic segment size estimation

SA11 segment	Migration distance (mm)	Segment size base pairs	Segment size Log_{10}
1	22	3302	3.52
2	28	2690	3.43
3	31	2591	3.41
4	32	2362	3.37
5	48	1611	3.21
6	55	1356	3.13
7	69	1104	3.04
8	70	1059	3.02
9	71	1062	3.03
10	87	751	2.88
11	96	667	2.82

PBV 21975/89	Migration distance (mm)	PBV segment size Log_{10}	PBV segment size base pairs	Total genome size (bp)
1A	30.5	3.4	2500	4230
1B	45	3.24	1730	

(data for Figure 5., see appendix for calibration curve Figure 69.)

The genome profiles of eight further strains of picobirnavirus are shown in Figure 6. These strains were detected in faecal samples screened between 1991 and 1993 and form part of the epidemiological studies described in this thesis (see R7). The sizes of the two genomic segments for each of the strains were estimated using the same procedure as for PBV 21975/89. Measurements for the eight strains were taken from Figure 6. and are shown in Table 6.

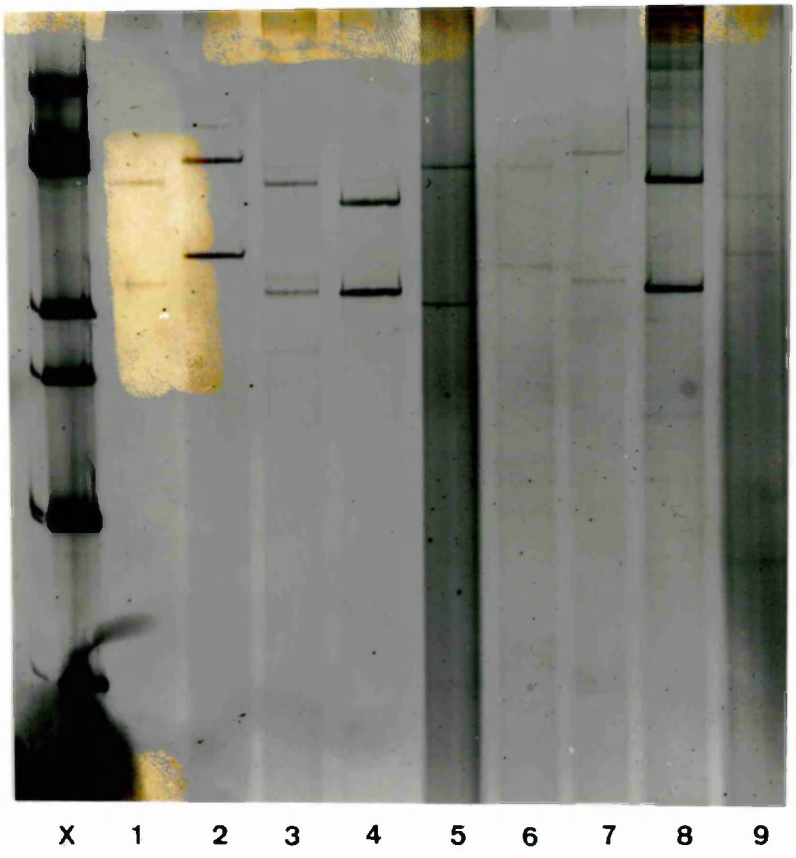


Figure 6. **PAGE gel demonstrating various genomic profiles of picobirnaviruses used to estimate genomic segment sizes** Lane X shows SA11 (size marker), lane 1 PBV 34/93, lane 2 PBV 734/93, lane 3 PBV 2915/93, lane 4 PBV 2916/93, lane 5 PBV 14030/93, lane 6 PBV 10964/93, lane 7 is 21975/89 (see Table 5.), lane 8 PBV 10494/92 and lane 9 3380/93. The origin of additional bands seen in lanes 2, 3, and, 4 were unknown , but they were not considered to be PBV-like.

Table 6. Determination of the size of eight strains of picobirnavirus

PBV strain & lane no.	Seg. No.	PBV segment size (bp)	Total size (bp)
1) 34/93	1	2410	4160
	2	1750	
2) 734/93	1	2540	4480
	2	1940	
3) 2915/93	1	2380	4110
	2	1730	
4) 2916/93	1	2240	3970
	2	1730	
5) 14030/93	1	2540	4220
	2	1680	
6) 10964/93	1	2540	4420
	2	1880	
*)			
8) 10494/92	1	2370	4200
	2	1750	
9) 3380/93	1	2330	4290
	2	1960	

* Lane 7) 21975/89, see Table 5.

An estimate of the total genome size for the PBV strains were calculated by adding the size of segment 1 and segment 2 for each strain. The values for the sizes of the PBV genomic segments are estimates and are accurate to approximately + or - 50 base pairs, which is the accuracy of the graph measurements (Table 6.). The PBV strain 21975/89 (Figure 5.) had a total genome size of 4230 bp and the characteristic PBV described by Pereira *et al.*, 1988a, was 4000 bp.

The genome sizes of seven PBV strains detected in HIV-infected patients (Figure 7.) were also estimated and compared to those PBV strains already shown. These PBV strains were examined as part of the epidemiology studies (see R7.d). The sizes of the genomic segments for the PBV strains in this group were estimated using the same procedure as used for the other strains (Table 7.).

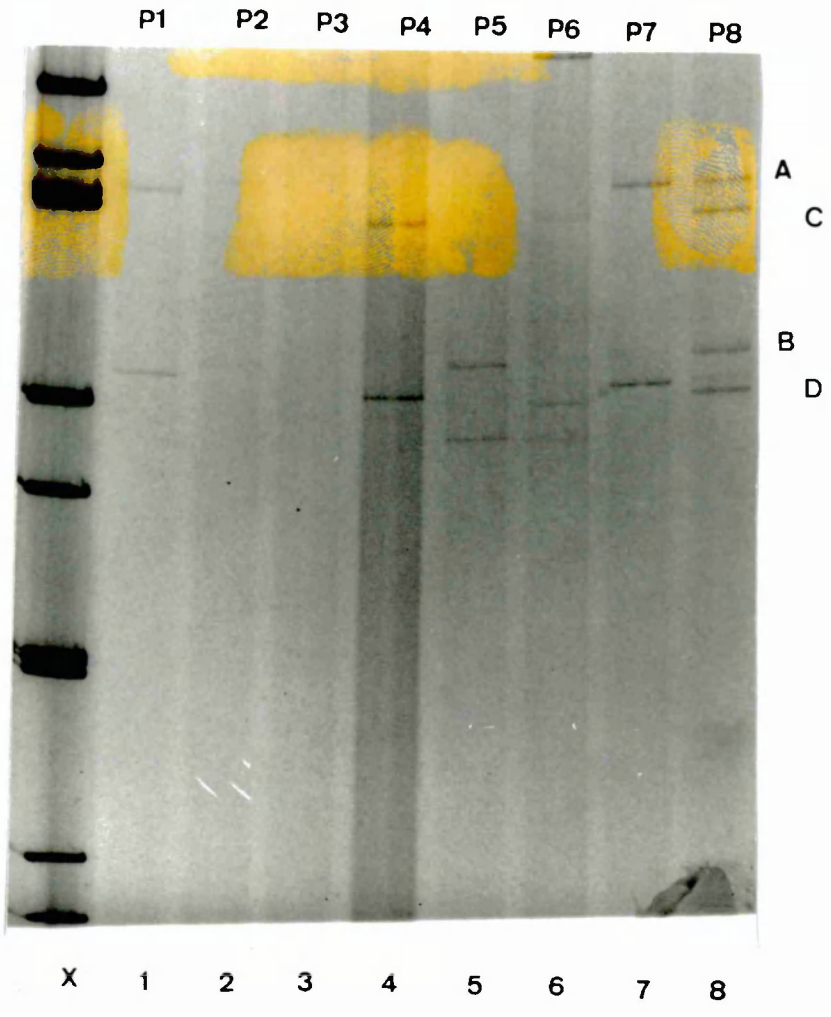


Figure 7. **Sizing of seven picobirnavirus strains detected in HIV-infected patients by PAGE**

Lane 1,2,4,6,7, and 8 show PBV strains P1, P2, P4, P6, P7, and P8. Lane 8 shows strain P8-1 (A & B) and P8-2 (C & D). In Lane 3 there was a very weak positive sample which does not show in the photograph and in lane 5 there is a strain with an atypical profile, P5 (see R16.a). Lane X shows SA11 (size marker).

Table 7. Sizes of the genomic segments of PBV strains identified in HIV-infected patients

PBV strain & lane no.	Segment No.	Segment size (bp)	Total genome size (bp)
1) P1	1	2510	4190
	2	1680	
2) P2	1	2570	4290
	2	1720	
4) P4	1	2340	3915
	2	1575	
6) P6	1	2360	3910
	2	1550	
7) P7	1	2570	4220
	2	1650	
8) P8-1	1	2630	4430
	2	1800	
P8-2	1	2470	4150
	2	1680	

(data from Figure 7.)

The total genome size for PBV strains from both groups are in the range 3900 to 4500 base pairs. In the outbreak patients group (Figure 6.) the PBV segment 1 genome sizes ranged from 2240 to 2540 base pairs and 1680 to 1960 base pairs for segment 2. In the HIV group (Figure 7.) the segment 1 genome sizes ranged from 2340 to 2630 base pairs and 1550 to 1800 for segment 2.

The average genome size for segment 1 (slower migrating band) amongst the 16 PBV strains described is thus approximately 2460 base pairs and for segment 2 (faster migrating band) it is 1740 base pairs. The analysis does not include strain P5 (Figure 7. lane 5) which has a unique profile with two segments of approximately 1750 and 1550 base pairs (total genome 3300 base pairs).

R3) Characterisation of PBV nucleic acid by nuclease digestions

The nature of the nucleic acid represented by the two PBV genomic segments was investigated using nuclease digestion. The nucleases used were as follows: RNase A which digests both dsRNA and ssRNA, RNase T1 which digests only ssRNA and RQ1 DNase which digests only DNA. Control nucleic acids used were as follows: rotavirus SA11 (dsRNA), yeast tRNA (ssRNA) and ØX174/Hae III (dsDNA) or 1Kb DNA marker were digested for comparison (see MM5). A PAGE gel demonstrating the effect of the different nucleases on the PBV strain 21975/89 nucleic acid and control nucleic acid is shown in Figure 8 and the data is shown in Table 8. PBV 21975/89 nucleic acid was only digested by RNase A, the same digestion profile as rotavirus SA11 which has a dsRNA genome, and therefore it was concluded that the two genomic segments of PBV 21975/89 are dsRNA. The control nucleic acids confirmed the specific activity of the nucleases (Figure 8., Table 8.). The digestion profile for PBV strain 10494/92 (Figure 9., Table 9.) confirms it to be to be dsRNA.

Table 8. **PBV (21975/89) nucleic acid digestion data**

Lane		Nucleic acid	Nuclease	Result
	1	21975/89	No nuclease	No digestion
	2	"	RNase A	Digestion
A	3	"	RNase T1	No digestion
	4	"	RQ1 DNase	No digestion
	5	SA11 (dsRNA)	No nuclease	No digestion
	6	"	RNase A	Digestion
B	7	"	RNase T1	No digestion
	8	"	RQ1 DNase	No digestion
	9	Yeast tRNA (ssRNA)	No nuclease	No digestion
	10	"	RNase A	Digestion
C	11	"	RNase T1	Digestion
	12	"	RQ1 DNase	No digestion
	13	ØX174/Hae III (dsDNA)	No nuclease	No digestion
	14	"	RNase A	No digestion
D	15	"	RNase T1	No digestion
	16	"	RQ1 DNase	Digestion

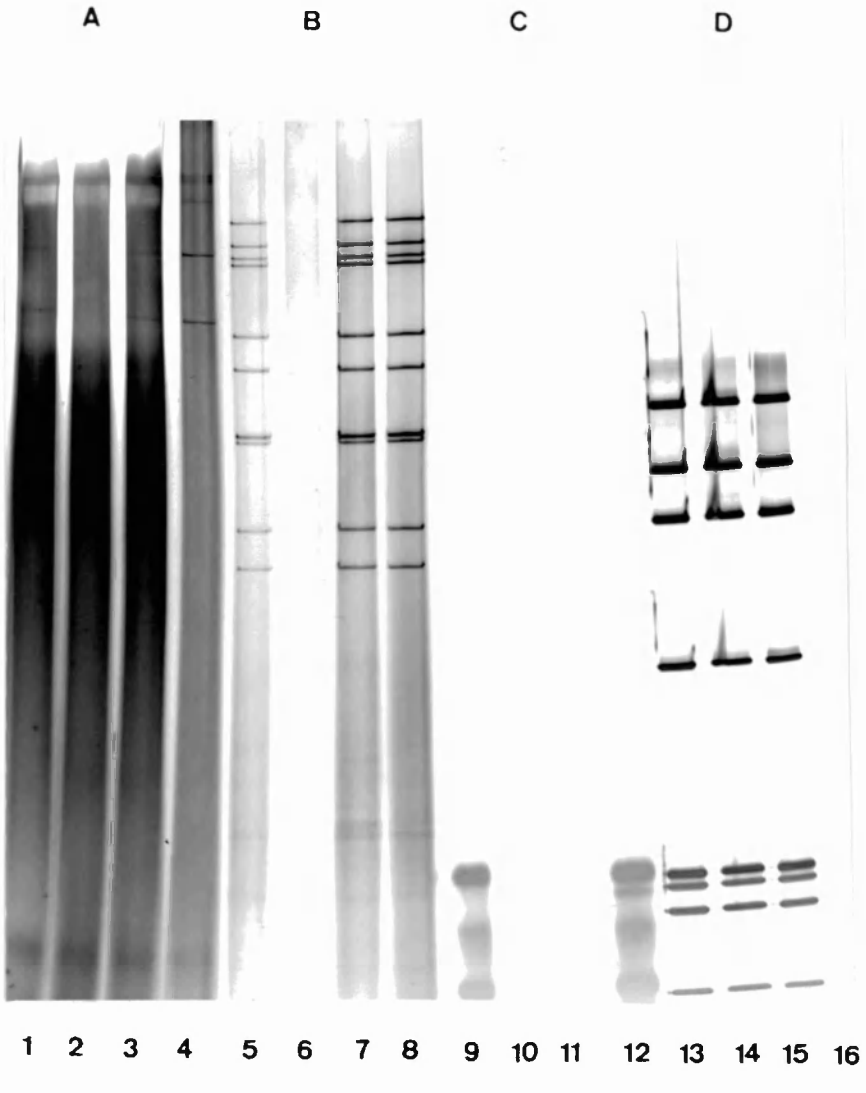


Figure 8. PAGE analysis of samples from nuclease digestion experiment with PBV strain 21975/89. Lanes 1 to 16 (see Table 8.)

Table 9. **PBV (10494/92) nucleic acid digestion data**

	Lane	Nucleic acid	Nuclease	Result
	1	SA11 (dsRNA)	No nuclease	No digestion
	2	"	RNase A	Digestion
A	3	"	RQ1 DNase	No digestion
	4	"	RNase T1	No digestion
	5	10494/92	No nuclease	No digestion
	6	"	RNase A	Digestion
B	7	"	RQ1 DNase	No digestion
	8	"	RNase T1	No digestion
	9	Yeast tRNA (ssRNA)	No nuclease	No digestion
	10	"	RNase A	Digestion
C	11	"	RQ1 DNase	No digestion
	12	"	RNase T1	Digestion
	13	1Kb ladder (dsDNA)	No nuclease	No digestion
	14	"	RNase A	No digestion
D	15	"	RQ1 DNase	Digestion
	16	"	RNase T1	No digestion

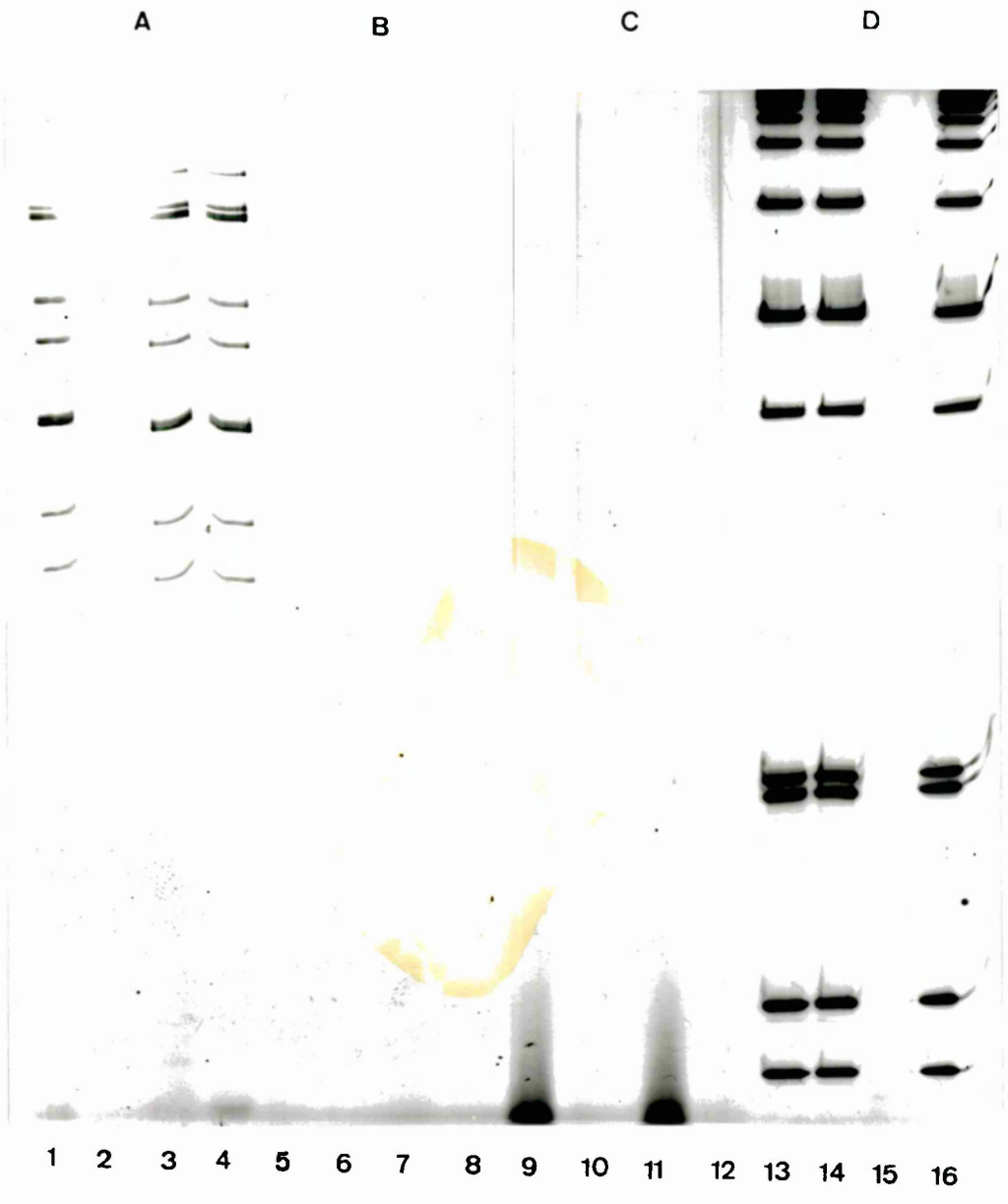


Figure 9. PAGE analysis of samples from nuclease digestion experiment with PBV strain 10494/92. Lanes 1 to 16 (see Table 9).

R4) Determination of buoyant density in caesium chloride (CsCl) of picobirnavirus particles from human faeces by PAGE analysis

Buoyant density determinations were possible on only a few samples as insufficient material was available to perform the procedure on other PBV samples.

Two PBV strains, 10492/92 and 10494/92, were analysed as described (MM6). Their buoyant density in CsCl were determined by PAGE analysis of individual fractions.

Strain 10492/92 showed two bands which peaked in staining intensity (Figure 10.) in fraction 8, which corresponded to a buoyant density of 1.385 g/ml in CsCl. Faint PBV bands were seen either side of the peak fraction. The PAGE analysis of the CsCl fractions from PBV strain 10494/92 demonstrated the PBV bands in fractions 5 to 11 (Figure 11.), with the most intensely stained bands in fraction 7 corresponding to a buoyant density of 1.38 g/ml in CsCl.

The PAGE gel of CsCl fractions from the purification of sample 21975/89 demonstrated the PBV bands in fraction 9 which had a corresponding buoyant density of 1.395 g/ml. The bands were very faint and the gel was not photographed.

The refractive index and buoyant density conversion table is shown in Table 28. (see appendix). The data obtained for the two PBV strains is shown in Table 10.

Table 10. **Data for buoyant density determination for PBV strains**

	10492/92				10494/92		
Fract. No.	RI	BD	PBV bands	Fract. No.	RI	BD	PBV bands
5	1.3675	1.3600		5	1.3675	1.3600	+
6	1.3690	1.3750		6	1.3685	1.3700	++
7	1.3700	1.3850	+	7	1.3695	1.3800	+++
8	1.3710	1.3850	++	8	1.3705	1.3900	++
9	1.3720	1.4050	+	9	1.3715	1.4000	+
10	1.3730	1.4150	+	10	1.3725	1.4100	+
11	1.3740	1.4250		11	1.3735	1.4200	+

Staining intensity of PBV bands: + = Weak, ++ = Medium, +++ = Strong (see Figure 10. and 11.).

RI = Refractive Index, BD = Buoyant Density

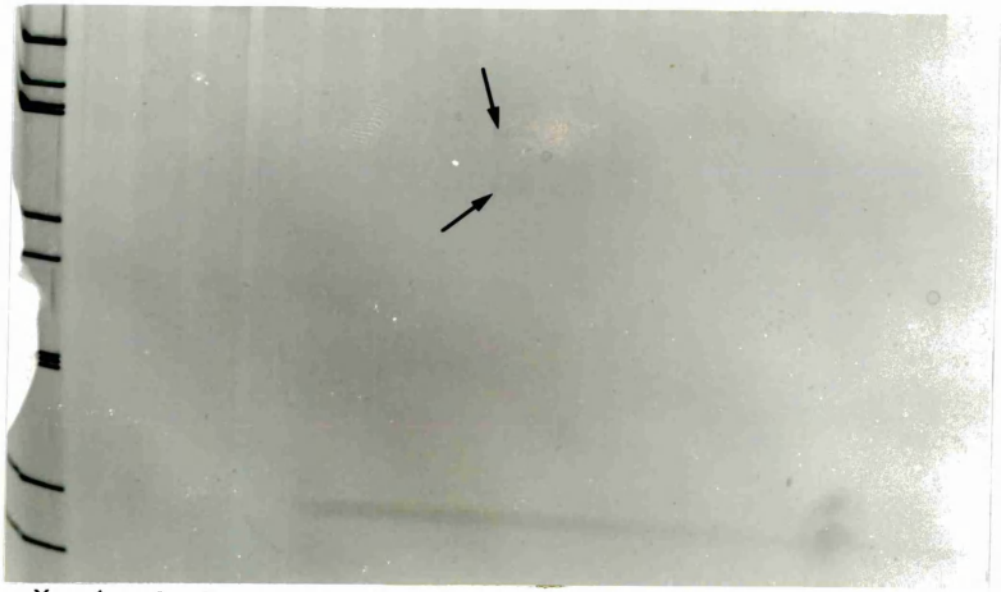


Figure 10. PAGE analysis of nucleic acid from CsCl fractions of sample 10492/92 Lanes 1 to 16 correspond to fraction 1 to 16 (see Table 11.). lane X is SA11.

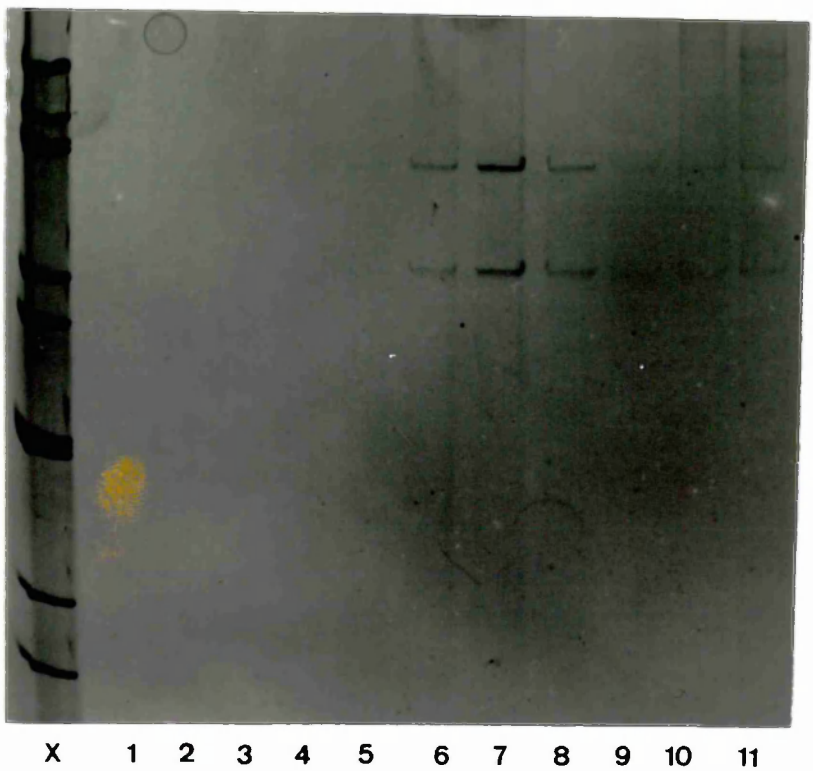


Figure 11. PAGE analysis of nucleic acid from CsCl fraction of sample 10494/92 Lanes 1 to 11 correspond to fractions 1 to 11 (see Table 10.). lane X is SA11

R5) Electron microscopy of picobirnavirus-like particles in human faeces

a) Electron microscopy of CsCl gradient purified human faeces positive for PBV by PAGE [D.Lewis, Leeds PHL]

Electron microscopy was performed on purified faecal samples (MM6) as described in MM7. No virus particles were seen in any of the fractions from samples 10492/92 and 10494/92, which contained PBV bands (Figure 10. and 11.) by PAGE analysis.
Electron microscopy was also performed on CsCl fractions from sample 21975/89 [by H.Appleton, VRD, CPHL], but no virus particles were seen.

b) Direct electron microscopy of human faecal samples positive for PBV by PAGE and negative for PBV by PAGE

Direct electron microscopy was performed on 35 faecal samples that were positive for PBV by PAGE analysis. In the PAGE positive PBV samples, 8/35 (23%) contained PBV-like particles, 2/35 (6%) had possible PBV-like particles and 25/35 (71%) were negative for PBV-like particles. Also faecal samples sent for routine screening (Leeds PHL and VRD) for viral agents of gastroenteritis were examined for the presence of PBV-like particles, five of which contained PBV-like particles but were subsequently demonstrated as PAGE negative.

This EM was performed by D.Lewis (DL- Leeds PHL) and H.Appleton (HA- VRD), all micrographs are at x 200,000 magnification (Figures 12., 13., and 14. DL; Figures 15., 16., and 17. HA)

Particles from 2 of the 8 PAGE positive samples (10494/92 and 3380/92 [Gallimore *et al.*, 1995a]) were examined in more detail. The PBV-like particles in 10494/92 measured at 39-41nm. The particles were seen both singly and in clumps. They were spherical with a smooth outline and in many of the particles a core could be distinguished, which was narrowly separated from the outer rim. No substructure could be seen in the core. The outer rim was approximately 3nm in thickness (Figure 12. and 13.). Similar PBV-like particles to those demonstrated in sample 10494/92 (Figures 12. and 13.) were also seen by EM in faecal sample 9122/91 (Figure 15.). PAGE analysis of this sample proved negative for the PBV genome. The PBV-like particles seen in faecal sample 3380/92 (Figure 14.) measured 35-38nm in diameter and were slightly smaller than the particles described earlier. They had a smooth rim and occurred in clumps. Although many particles appeared to have some surface structure, this was never clearly defined. The faecal sample 3380/92 was PAGE positive for PBV. A further two faecal samples 10876/92 and 3378/92 contained PBV-like particles by EM, but PAGE analysis failed to demonstrate PBV genomic bands. There were a large group of particles seen in sample 10876/92 (Figure 16.) which appear to have a similar particle morphology to those seen in sample 3380/92 (Figure 14.) and sample 3378/92 (Figure 17.).

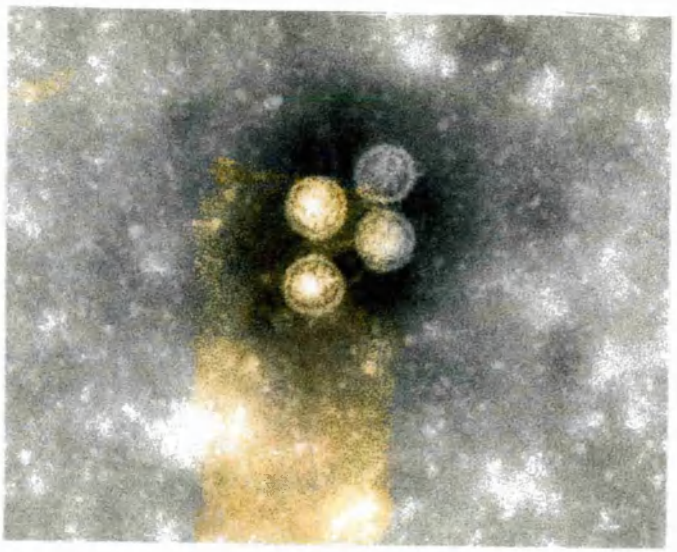


Figure 12. **Electron micrograph of four PBV-like particles human faecal sample 10494/92.** Mag. x200,000 (courtesy of D.Lewis)

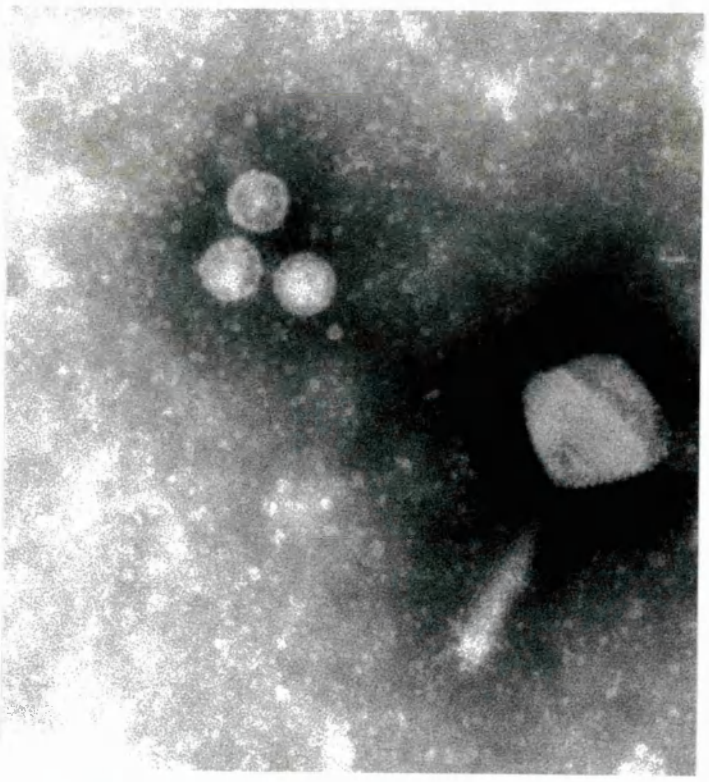


Figure 13. **Electron micrograph of three PBV-like particles in human faecal sample 10494/92.** Mag. x200,000 (courtesy of D.Lewis)

Figure 14. **Electron micrograph of PBV-like particles in human faecal sample 3380/92.** Mag. x200,000 (courtesy of H.Appleton)

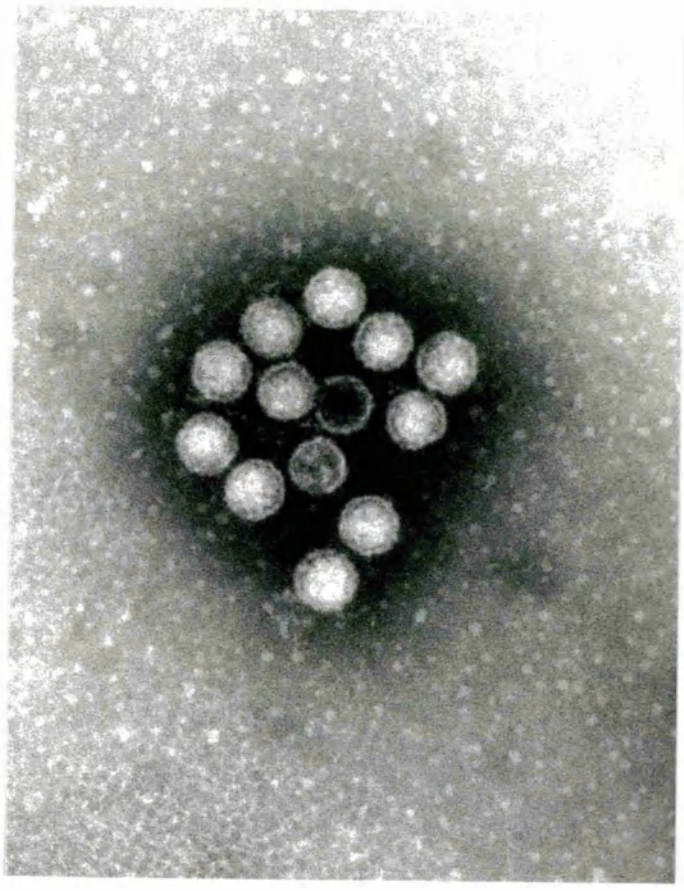


Figure 15. **Electron micrograph of PBV-like particles in PAGE negative human faecal sample 9122/91.**
The PBV-like particles form a large group and there are two particles in the centre, one which appears to be an empty particle and one which is partially empty.
Mag. x200,000 (courtesy of D.Lewis)

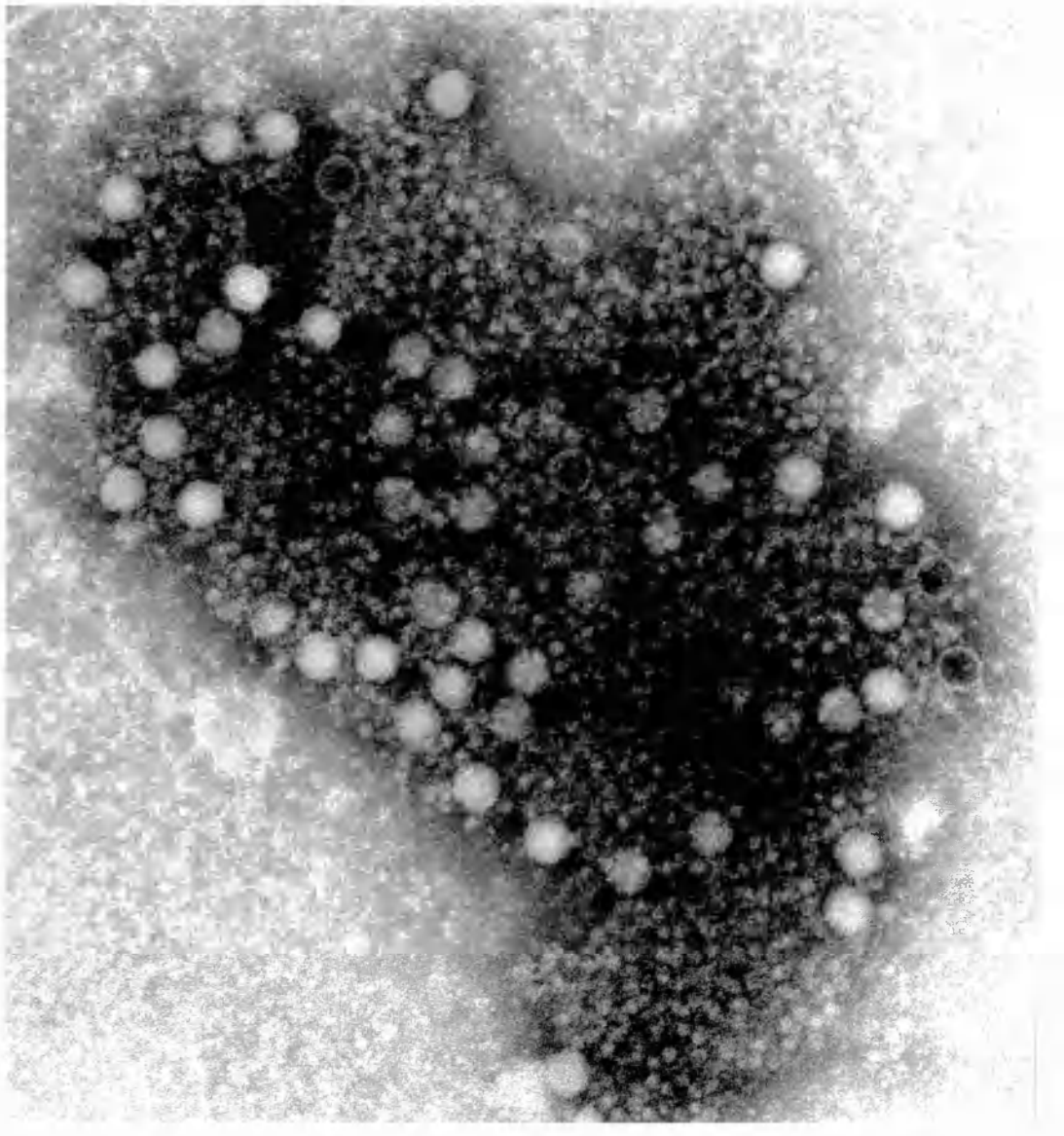


Figure 16. **Electron micrograph of PBV-like particles in PAGE negative human faecal sample 10876/92.**
Mag. x200,000 (courtesy of H.Appleton)

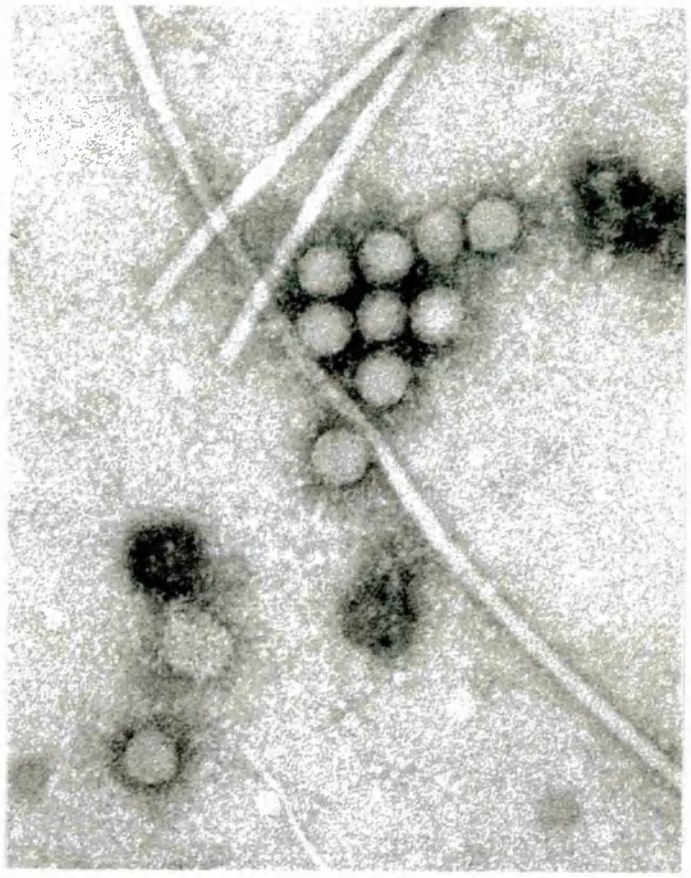


Figure 17. **Electron micrograph of PBV-like particles in PAGE negative human faecal sample 3378/92.**
Mag. x200,000 (courtesy of H.Appleton)

R6) Optimisation of various parameters of the 'Boom' method for extraction of dsRNA from faeces

The technique used originally in work described in this thesis to extract nucleic acid from faeces was a standard phenol/ chloroform and ethanol precipitation method (MM2). Subsequently the guanidinium thiocynate/silica method ('Boom' method) and RNAzol B were used (MM2). The optimisation of various parameters of the 'Boom' method is described below.

The optimisation of the 'Boom' method was performed on group A rotaviruses in faecal extracts as a model for dsRNA, as only small quantities of PBV positive faeces were available.

a) Estimation of the quantity of SA11 virus extracted by the 'Boom' method required as a control in a PAGE gel

Standard conditions described for the 'Boom' method were used (see MM2). Tissue culture grown SA11 was used in the extraction procedure in 50, 100, 200, 300, 400 and 500ul volumes of tissue culture fluid (TCF), and estimated to be approximately equivalent to 5, 10, 20, 30, 40 and 50 ng SA11 dsRNA respectively (Berry and Samuel, 1982). It shows that 100 - 200ul of TCF (10 to 20 ng) is suggested as a suitable volume to use as an extraction control and molecular weight marker (Figure 18.).

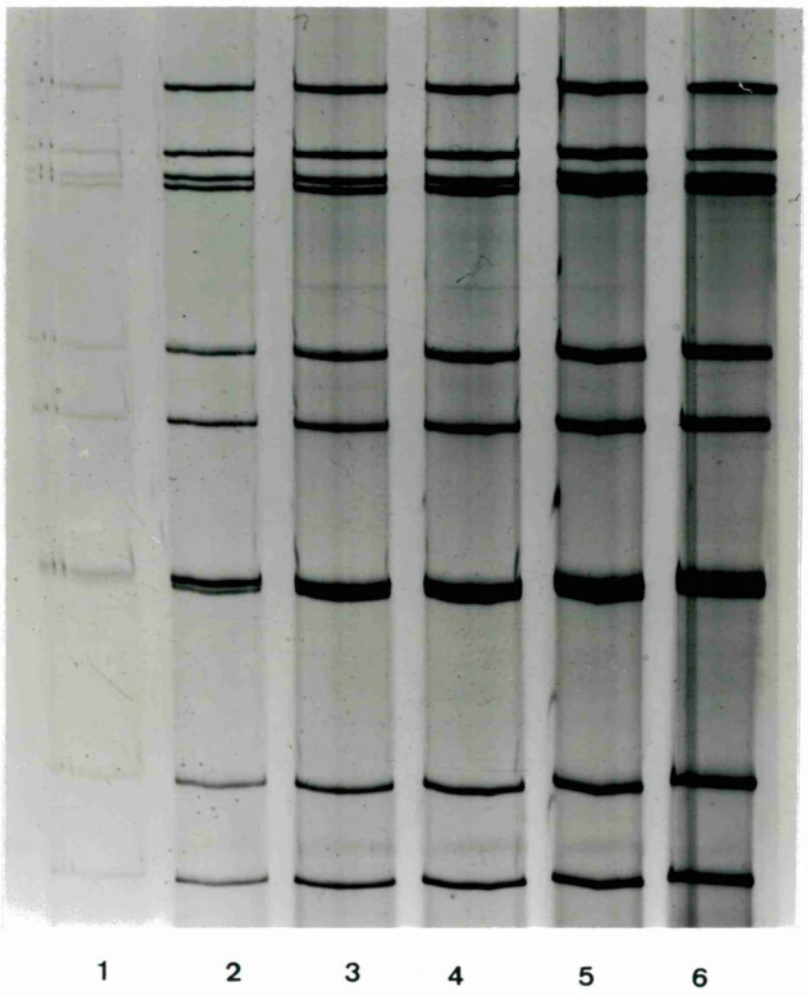


Figure 18. **PAGE analysis of the dsRNA from titrated SA11 using the 'Boom' method of nucleic acid extraction**
Lane 1, 2, 3, 4, 5 and 6 correspond to 50, 100, 200, 300, 400 and 500ul of SA11 TCF. Equivalent to 5, 10, 20, 30, 40, and 50 ng SA11 dsRNA.

b) **Effect of elution temperature and elution volume on recovery of dsRNA by the 'Boom' method**

Two faecal rotaviruses were used to see if there was any difference in elution efficiency between the use of the standard elution temperature of 56^{O}C and the slightly increased temperature of 60^{O}C and to determine what effect increasing the elution volume from 50 to 500ul had on the recovery of dsRNA.

It can be seen in Figure 19. that the standard conditions of eluting nucleic acid from silica at 56^{O}C for 15 min was satisfactory and there was no additional recovery of nucleic acid if the elution temperature was increased to 60^{O}C or the elution volume increased from 50ul to 100, 300 or 500ul.

c) **Effect of varying the volume of silica on recovery of dsRNA**

The quantity of dsRNA recovered from a faecal rotavirus sample using different volumes of silica in the 'Boom' method was determined by PAGE analysis. The results showed that there was no difference in the yield obtained when using quantities of silica ranging from 5ul to 40ul (Figure 20.).

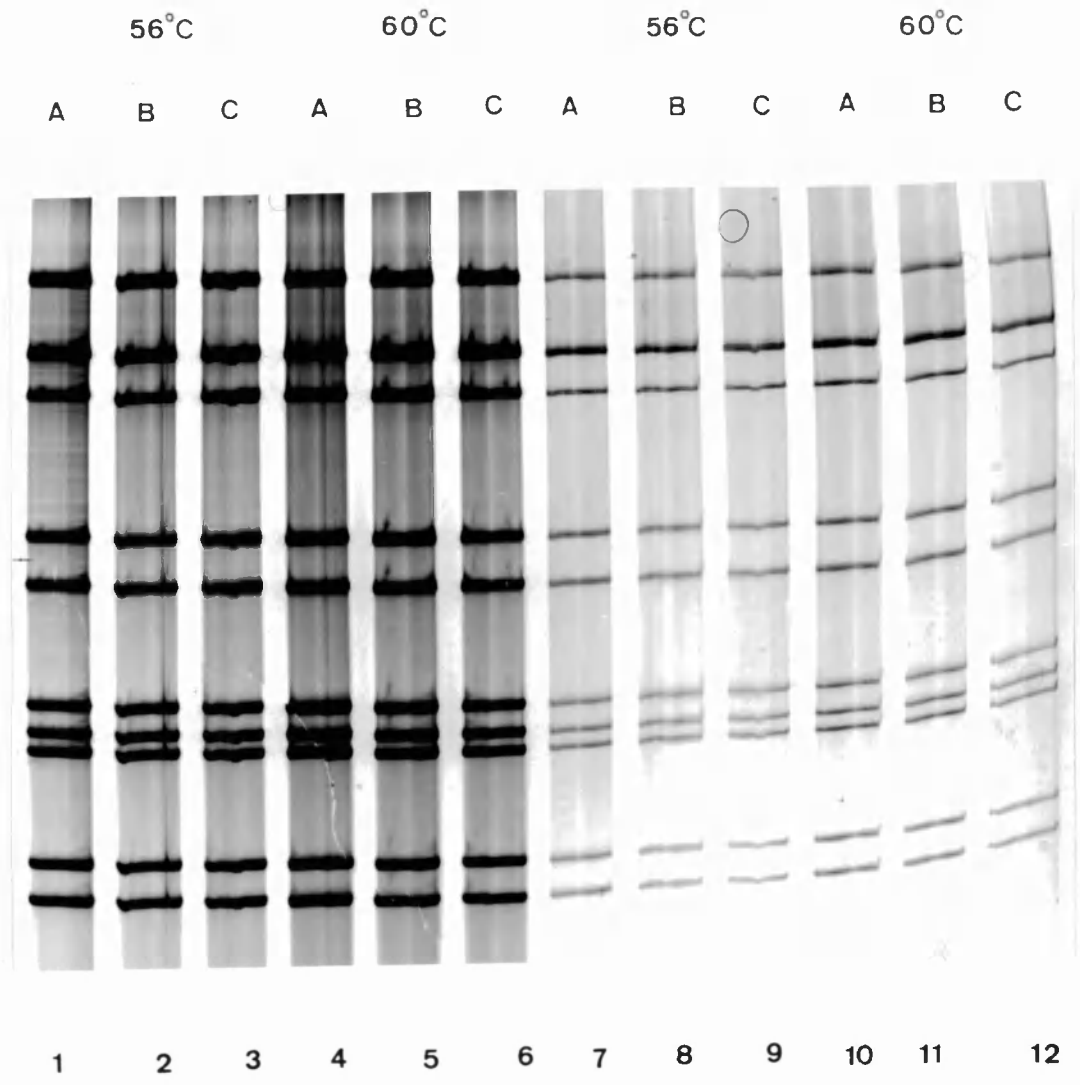


Figure 19. Effect of elution temperature and volume on recovery of dsRNA by the 'Boom' method as analysed by PAGE

Lane 1 to 6 shows a strongly positive group A rotavirus sample (16471/90) and lane 7 to 12 shows a weaker positive group A rotavirus sample (14474/90).

Samples A, B, and C are 100ul, 300ul and 500ul elute volumes respectively.

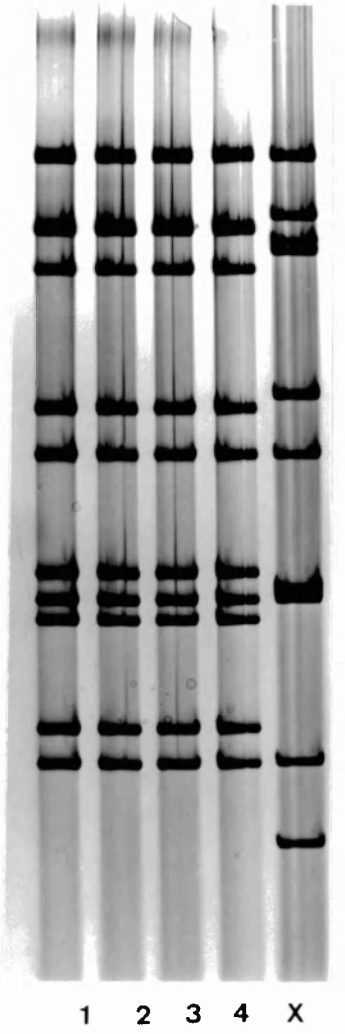


Figure 20. **Effect of varying the volume of silica on recovery of dsRNA using the 'Boom' method as analysed by PAGE**
Lane 1 5ul silica, lane 2 10ul, lane 3 20ul, lane 4 40ul. Lane X is SA11 size marker

d) Effect of successive elutions on the recovery of dsRNA by the 'Boom' method

This experiment investigated the quantity of dsRNA recovered by one elution and then subsequent elutions from the same sample. It can be seen in Figure 21., that the majority (approximately 80%) of dsRNA was recovered in the first elution. More nucleic acid, however, could be recovered if further elutions were performed.

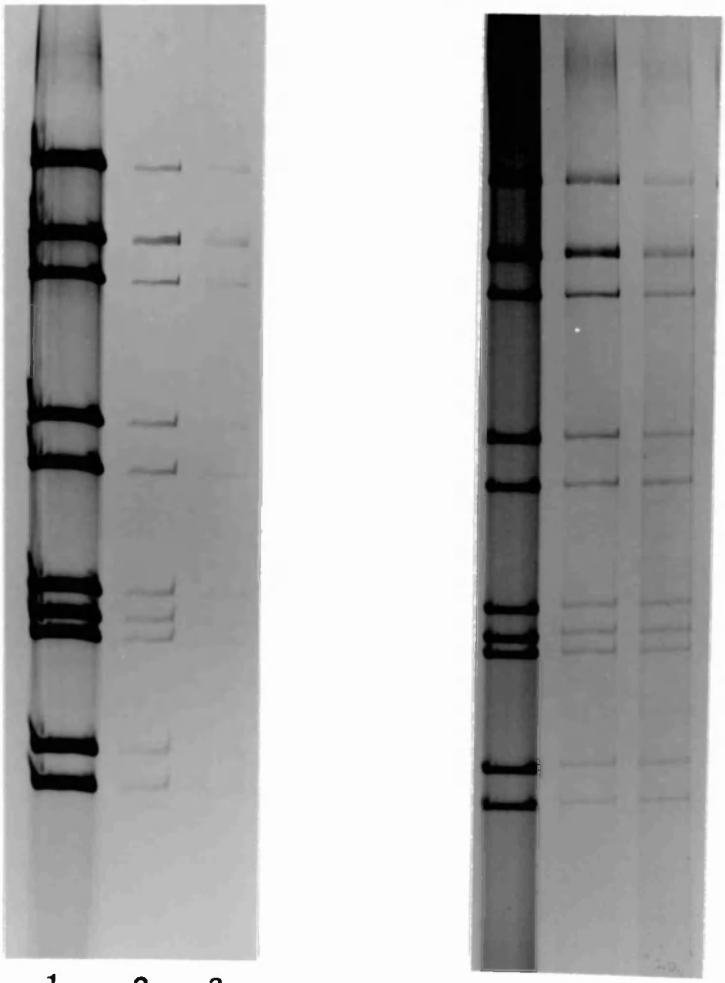


Figure 21. **Effect of successive elutions of dsRNA from silica used in the 'Boom' method**

Lane 1 shows rotavirus 16471/90 first elution, lane 2 second elution, lane 3 third elution. Lanes 4, 5 and 6 shows the same using rotavirus 14474/90.

SECTION 2. Human picobirnavirus epidemiology

In Section 2, the information that was obtained from the human PBV (HPBV) characterisation studies enabled some preliminary epidemiological studies to be undertaken.

R7) Epidemiology of picobirnaviruses in humans

a) Gastroenteritis outbreaks 1982-89

b) " " 1991-93

c) Hospitalised patients with and without gastroenteritis as the main presenting illness

d) HIV-infected patients with and without diarrhoea

e) Sporadic cases of gastroenteritis

R7) Epidemiology of picobirnaviruses in humans

Epidemiological studies were initiated to investigate the prevalence of picobirnaviruses in human faeces in the UK. The faecal samples were screened by PAGE analysis for the presence of PBV genomic bands. The studies were divided into various patient groups and all the samples were collected between 1982 and 1993.

a) Gastroenteritis outbreaks 1982-89

Faecal samples submitted to VRD for virological investigation from gastroenteritis outbreaks during 1982 to 1989 were investigated.

Study population

These faecal samples were stored at -40^{o}C and were screened retrospectively. They comprised 350 samples from 45 outbreaks. The

ages and age distribution of of patients were grouped as follows: children (0 to 17 years), 11.7%; adults (18 to 64 years), 31.1%; and elderly (over 65 years), 57.1%.

The places where outbreaks occurred were categorised as follows: geriatric wards (GW), old peoples homes (OPH), hotels and restaurants (HR), hospital wards, general (HWG), and schools (SCH). The hotels and restaurants group also includes any catered function where an outbreak had been food-associated. The hospital wards that were termed 'general' were wards that had adult patients that were not classed as geriatric.

The samples had been originally tested by electron microscopy (EM) and subsequently by PAGE for the presence of viruses. The EM and PAGE results are shown in Table 11. and Figure 23. Six groups of viruses were found: small round structured viruses (SRSV), rotaviruses (RV), small round particles (SRP), picobirnaviruses (PBV), and coronaviruses (CV). These viruses were diagnosed by EM, with the exception of PBV, which were only diagnosed by PAGE.

PBV detection

PAGE analysis revealed 12 of 350 samples (3.5%) to contain PBV. Six of 125 samples (5%) from geriatric wards were positive, 5/75 (7%) from old peoples homes, 0/63 from hotels and restaurants, 0/46 from hospital wards, general and 1/41 (2.4%) from schools (Figure 22.).

The distribution of PBV by patient age are as follows: elderly 11 PBV in 200 samples (5.5%), adults 0/109 (0%), and children 1/41 (2.4%). Of the picobirnaviruses detected in the 1982-89 outbreak samples 11/12 (92%) were detected in elderly patients and 1/12 (8%) in children (Figure 27.).

The distribution of patients by place of outbreak, age, and EM and PAGE results are shown in Table 11.

Table 11. **Outbreak samples 1982-89, place, age of patients and results of EM and PAGE**

Date of outbreak	Place of Outbreak	Age	No. of Spec's	EM (No.)	PBV (No.) by PAGE
1982	Convalescent home	Elderly	4	Neg	Neg
"	Old peoples home	"	14	SRP (1)	"
"	School	Children	6	Neg	PBV (1)
1984	Geriatric ward	Elderly	13	RV (5)	Neg
"	Hospital wards	Baby	10	SRSV (1)	"
		Elderly		SRP (1)	
				SRSV (2)	
"	Psychogeriatric ward	Elderly	7	RV (4)	"
"	Hospital ward	Adults	6	Neg	"
"	Hotel, restaurant	Adults	4	"	"
1985	Geriatric ward	Elderly	8	"	"
1986	"	"		RV (1)	"
				SRP (2)	
"	Hotel, restaurant	Adults	3	SRP (2)	"
				SRSV (3)	
"	School	Child	1	SRSV (1)	"

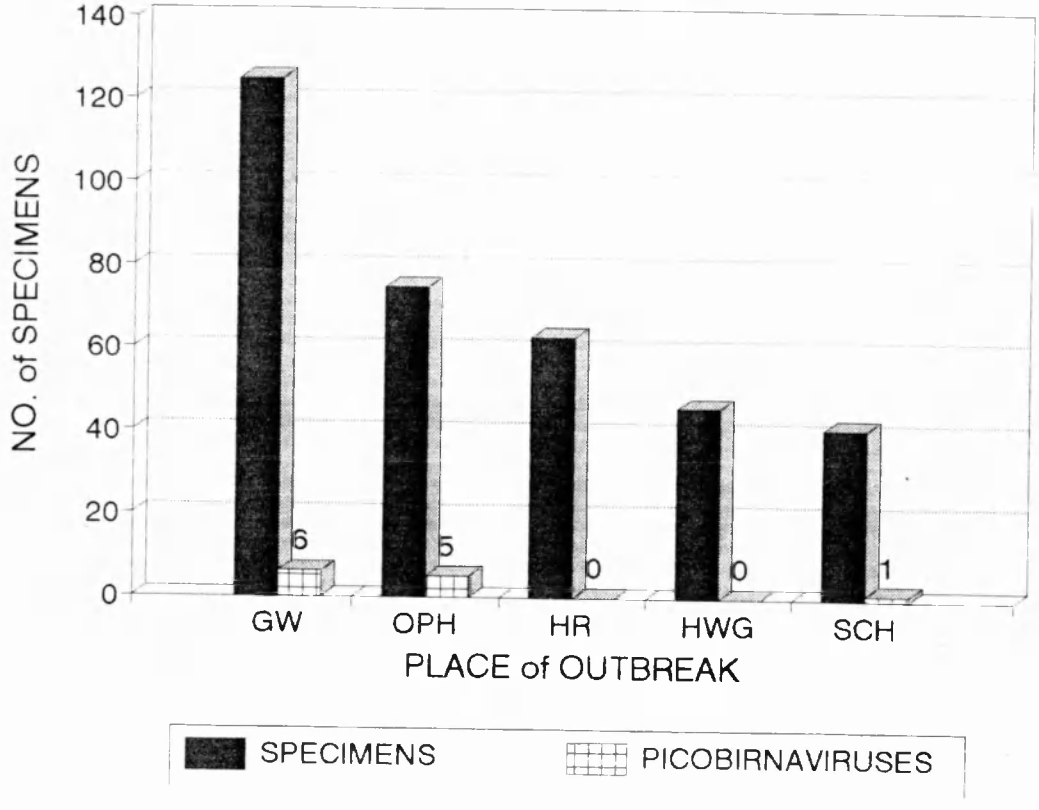
Table 11. Cont'd.

Date of outbreak	Place of Outbreak	Age	No of Spec's	EM (No.)	PBV (No.) by PAGE
1986	Geriatric ward	Elderly	8	RV (3) SRP (2)	"
"	Hospital, mentally ill	Adults	8	SRP (1)	"
1987	School	Children	5	SRSV (1) CV (2)	"
"	Hotel, restaurant	Adults	17	Neg	"
"	Geriatric ward	Elderly	13	SRSV (1)	PBV (1)
"	Hotel, restaurant	Adults	10	SRSV (1)	"
"	Old peoples home	Elderly	10	Neg	"
	"	"	3	"	
"	Geriatric ward	"	8	SRSV (1)	
"				CV (1) SRP (2)	
	"	"	5	Neg	"
"	Hotel, restaurant	Adults	12	SRSV (1) CV (1) SRP (1)	"
"	Old peoples home	Elderly	6	Neg	"
"	Geriatric ward	"	7	"	"
"	Old peoples home	"	3	"	"
	"	"	6	SRSV (1)	"
"	School	Children	14	SRSV (4)	"

Table 11. **Cont'd.**

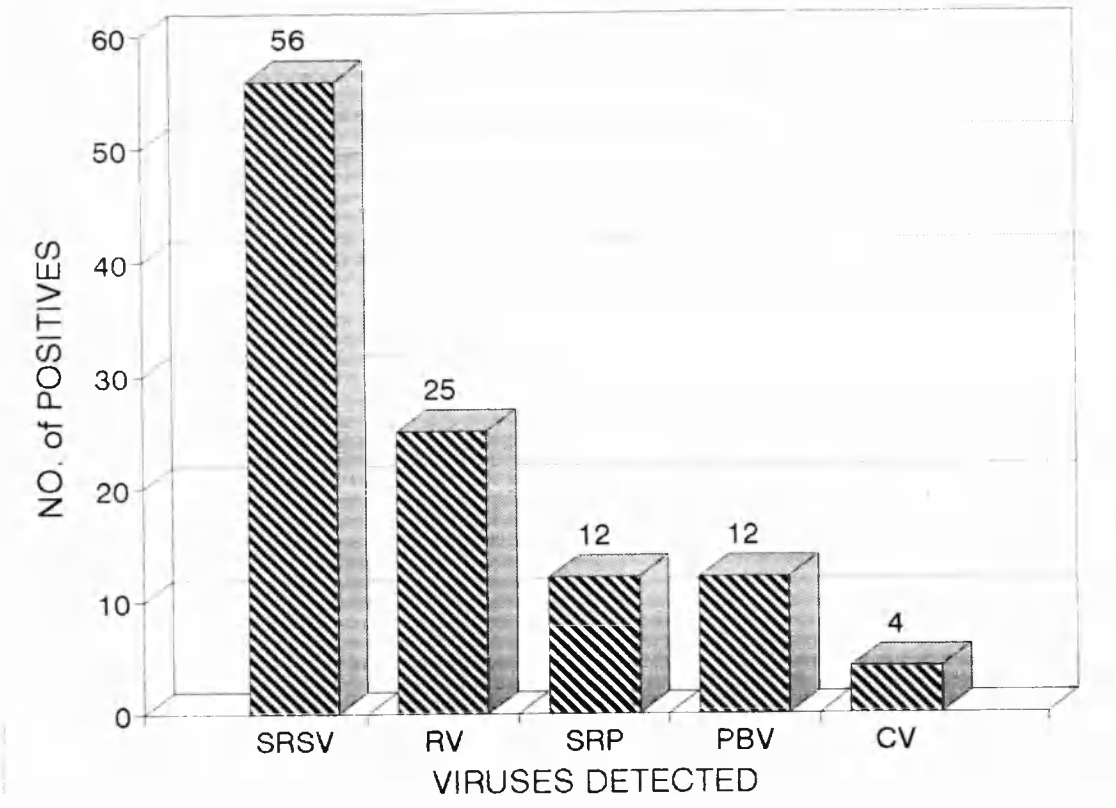
Date of outbreak	Place of Outbreak	Age	No of Spec's	EM (No.)	PBV (No.) by PAGE
1987	Old peoples home	Elderly	6	Neg	PBV (1)
"	Geriatric ward	"	6	"	PBV (1)
"	Old peoples home	"	8	SRSV (2)	PBV (2)
1988	Geriatric ward	"	6	Neg	Neg
"	School	Children	8	RV	"
"	Hotel, restaurant	Adults	7	SRSV (2)	"
"	Hospital ward	Adults	9	SRSV (5)	"
"	Geriatric ward	Elderly	8	SRSV (3)	PBV (2)
"	Geriatric ward	Elderly	4	SRSV (4)	PBV (1)
"	Old peoples home	"	10	SRSV (6)	PBV (1)
"	"	"	5	SRSV (4)	Neg
1989	School	Children	7	RV (4)	"
"	Geriatric ward	Elderly	13	SRSV (4)	PBV (1)
"	Hotel, restaurant	Adults	8	SRSV (1)	Neg
"	"	"	2	Neg	"
"	Geriatric ward	Elderly	11	SRSV (3)	Neg
"	Old peoples home	"	13	SRSV (6)	PBV (1)

Figure 22. Distribution of outbreak samples and PBV in the 1982-89 specimens by place of outbreak



GW = Geriatric wards, OPH = Old peoples' homes, HR = Hotels and restaurants, HWG = Hospital wards, general, SCH = Schools

Figure 23. Viruses detected in outbreak samples 1982-89 by EM and PAGE



SRSV = Small round structured virus, RV = Rotavirus

SRP = Small round particle, PBV = Picobirnavirus (PAGE only).

CV = Coronaviruses

b) Gastroenteritis outbreaks 1991-93

In this study 334 faecal samples from 47 outbreaks and 29 sporadic cases were stored at 4^{o}C prior to analysis. The samples were extracted using the 'Boom' method and analysed by PAGE for the presence of PBV.

Study population

The age distribution of the patients were as follows: elderly 166/334 (50%), adults 134/334 (40%), children 28/334 (8%) and unknown 6/334 (2%).

The categories of place where the outbreak occurred in are the same as those for the outbreak samples 1982-89. However, there is one additional category: sporadic cases (SPR), which are single patients who develop gastroenteritis and who are not part of a larger group of affected patients (ages of some of these are unknown).

The viral agents detected by EM are the same as for the 1982-89 and are shown in Figure 25.

PBV detection

PAGE analysis showed 30 of 334 (9%) samples were positive for PBV. Picobirnaviruses were detected in 10/98 (10%) of samples from geriatric wards, 15/95 (16%) from hotels and restaurants, 1/64 (2%) from old peoples homes, 1/25 (4%) from hospital wards, general, 1/23 (4%) from schools, and 2/29 (7%) from sporadic cases which were not part of a larger outbreak (Figure 24.).

These detection rates are compared to outbreak samples from 1982-89 (Figure 26.)

The distribution of PBV by patient age are as follows: elderly 12 PBV in 166 samples (7%); adults 16/134 (12%); and children 1/28 (4%);

and unknowns 1/6 (17%). Of the picobirnaviruses detected in the 1991-93 outbreak samples 12/30 (40%) were detected in elderly patients, 16/30 in adults (53%), 1/30 (3.5%) in children and 1/30 in age unknown (3.5%) and are compared to the 1982-89 samples (Figure 27.).

The distribution of patients by year, place of outbreak, age, and EM and PAGE results are shown in Table 12.

Table 12. **Outbreak samples 1991-93, place, age of patients and results of EM and PAGE**

Date of outbreak	Place of Outbreak	Age	No. of Spec's	EM (No.)	PBV (No.) by PAGE
1991	Geriatric ward	Elderly	6	SRSV (3)	Neg
"	"	"	2	Neg	"
"	Hotel, restaurant	Adults	4	SRSV (2)	"
"	Geriatric ward	Elderly	5	SRSV (1)	"
"	Old peoples home	"	2	RV (2)	"
"	Hotel, restaurant	Adults	8	SRSV (1)	PBV (2)
"	"	"	4	SRSV (2)	Neg
"	Hospital ward	Children	3	Neg	"
"			2	SRSV (1)	"
"			4	Neg	"
"	Hotel, restaurant	Adults	10	Neg	PBV (1)
"	Old peoples home	Elderly	7	SRSV (1)	Neg
"	"	"	4	SRSV (4)	"
"	Hospital ward	Children	6	Neg	PBV (1)
"	School	"	5	SRSV (1)	Neg
"	Hospital ward	Adult	2	SRSV (2)	Neg

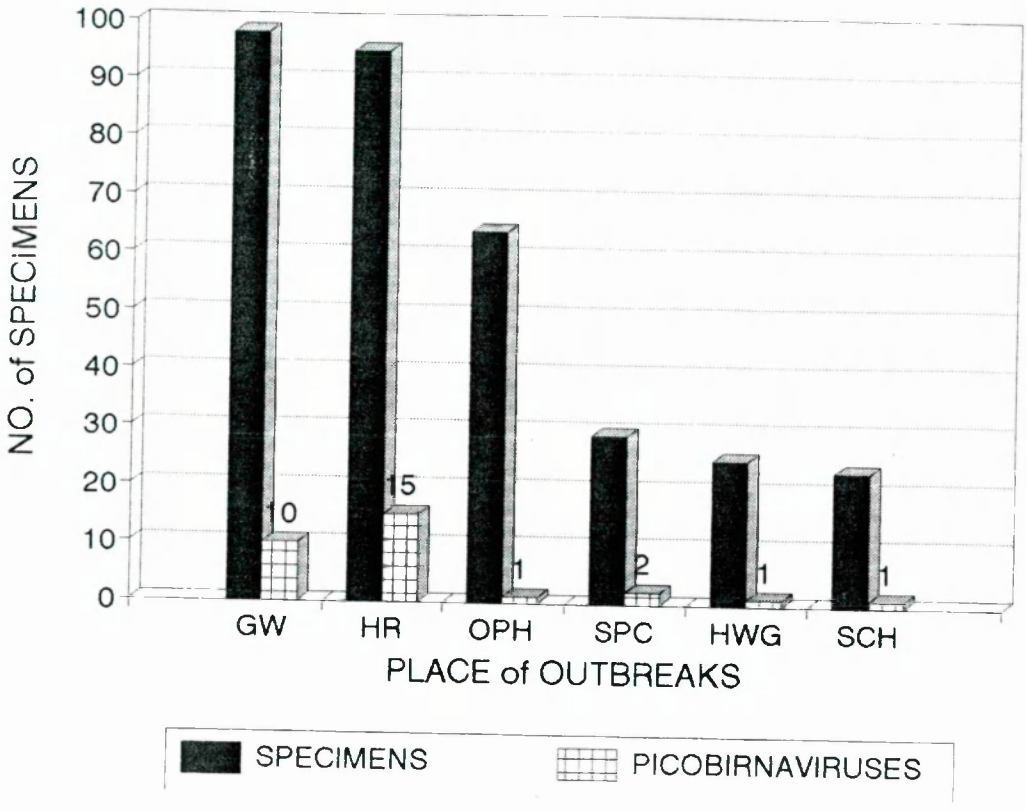
Table 12. **Cont'd**

Date of outbreak	Place of Outbreak	Age	No. of Spec's	EM (No.)	PBV (No.) by PAGE
1991	Geriatric ward	Elderly	12	Neg	PBV (1)
"	School	Children	9	SRSV (2)	PBV (1)
"	Sporadic	Unknown	6	Neg	PBV (1)
"	Hotel, restaurant	Adults	6	"	Neg
"	Sporadic	"	1	"	"
"	Old peoples home	Elderly	2	"	"
"	Geriatric ward	"	14	SRSV (2)	PBV (2)
"	Sporadic	Adult	8	Neg	Neg
"	Sporadic	Elderly (4) Adult (3) Child (5)	12	"	PBV (1)
1992	Geriatric ward	Elderly	7	SRSV (1)	PBV (1)
"	Hotel, restaurant	Adult	6	Neg	PBV (1)
"	"	"	1	SRP (1)	"
"	"	"	5	Neg	"
"	Geriatric ward	Elderly	3	"	"
"	"	"	19	"	PBV (2)
"	Old peoples home	"	15	SRSV (4)	Neg
"	Geriatric ward	"	2	Neg	"
"	Hotel, restaurant	Adult	4	"	"
"	"	"	3	"	"
"	"	"	6	SRSV (4)	"
"	Old peoples home	Elderly	5	SRSV (2)	"
"	"	"	7	SRSV (3)	"

Table 12. Cont'd

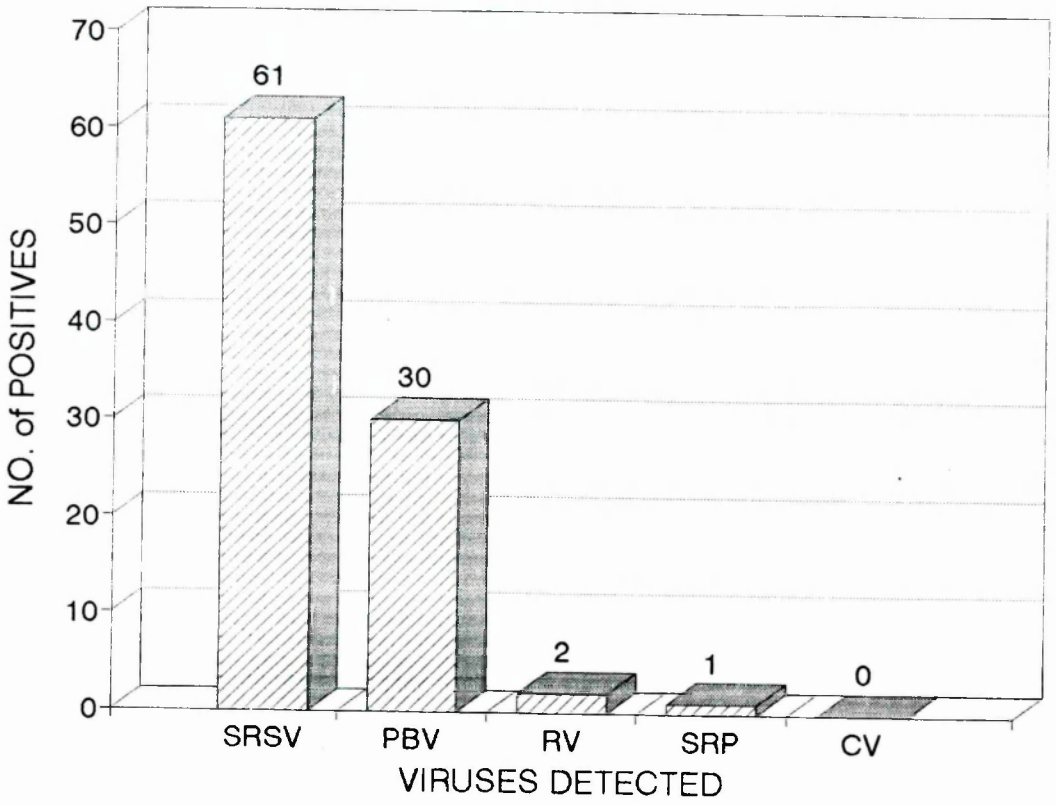
Date of outbreak	Place of Outbreak	Age	No. of Spec's	EM (No.)	PBV (No.) by PAGE
1992	Old peoples home	Elderly	9	SRSV (5)	Neg
"	Hospital ward	Adult	8	Neg	"
"	Hotel, restaurant	"	3	"	"
"	"	"	3	"	PBV (2)
1993	"	"	13	SRSV (3)	PBV (4)
"	Geriatric ward	Elderly	6	SRSV (1)	PBV (1)
"	Hotel, restaurant	Adult	19	SRSV (2)	PBV (5)
"	Old peoples home	Elderly	3	Neg	Neg
"	Geriatric ward	"	3	SRSV (2)	"
"	"	"	4	Neg	PBV (1)
"	"	"	8	SRSV (5)	PBV (2)
"	School	Adult	6	SRSV (1)	Neg
"	Sporadic	"	1	Neg	"
"	"	"	1	"	"
"	Geriatric ward	Elderly	7	"	"
"	School	Children	3	SRSV (2)	"
"	Old peoples home	Elderly	10	Neg	PBV (1)

Figure 24. Distribution of outbreak samples and PBV in the 1991-93 specimens by place of outbreak



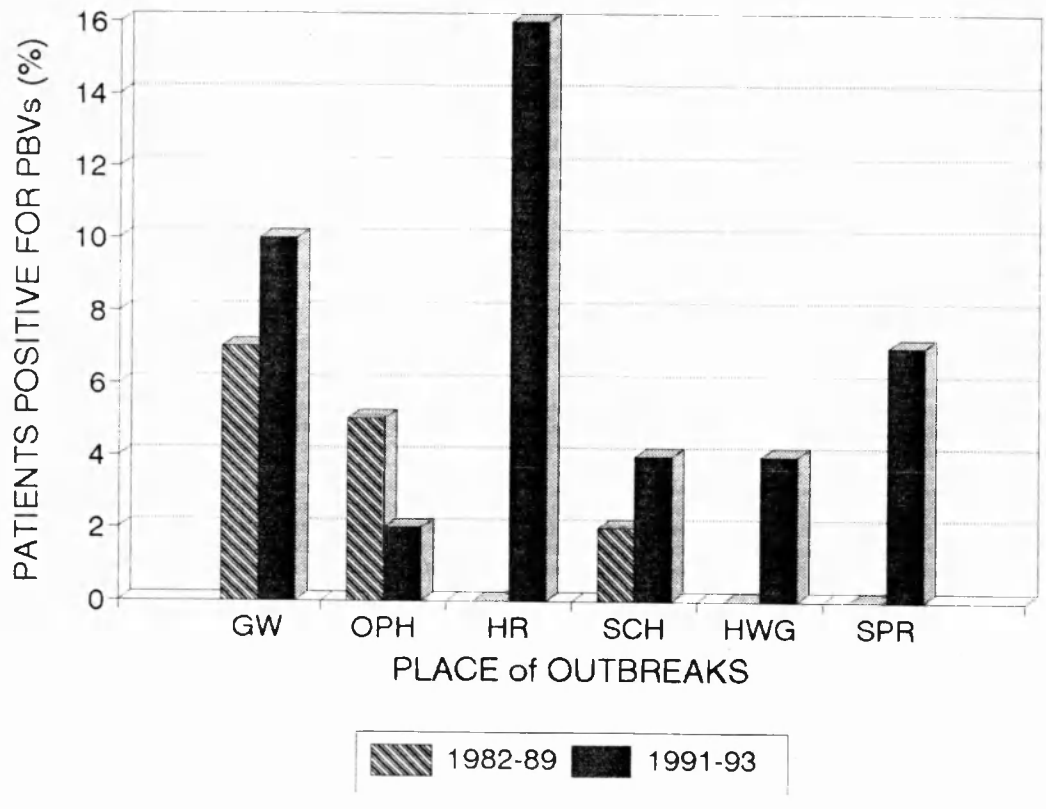
GW = Geriatric wards, OPH = Old peoples homes, HR = Hotels and restaurants, HWG = Hospital wards, general, SCH = Schools, SPC = Sporadic cases (not associated with an outbreak)

Figure 25. Viruses detected in outbreak samples 1991-93 by EM and PAGE



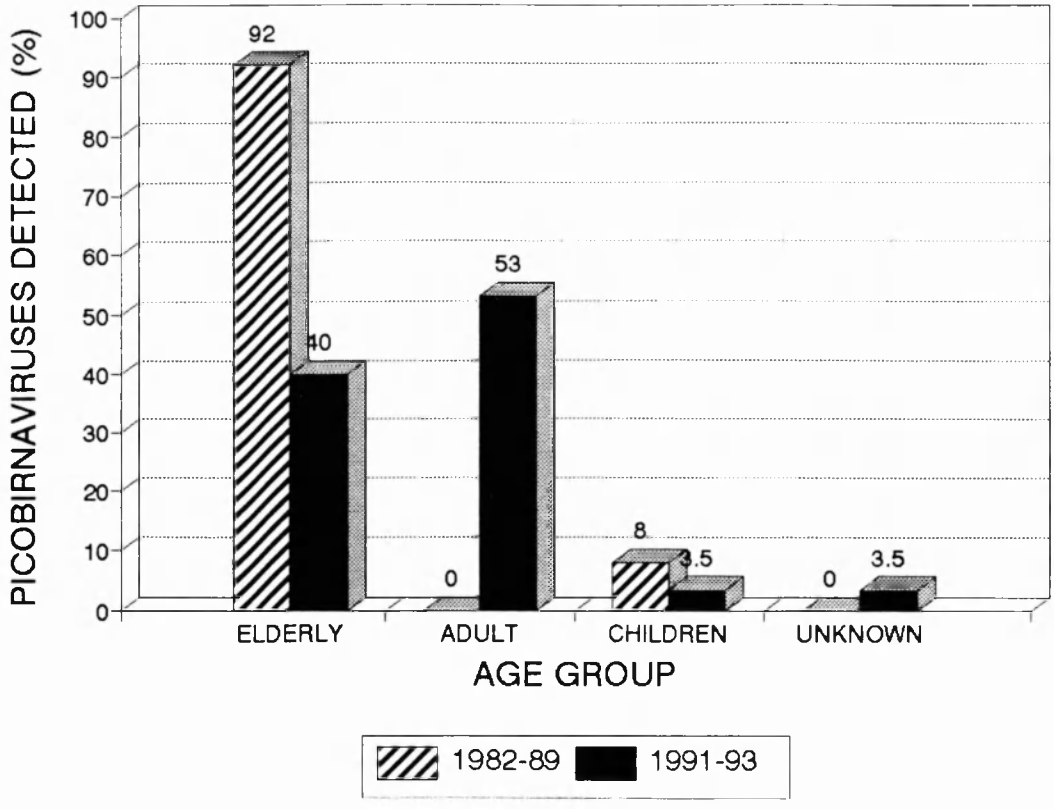
SRSV = Small round structured virus, RV = Rotavirus, SRP = Small round particle, PBV = Picobirnavirus (PAGE only), CV = Coronaviruses

Figure 26. Comparison of PBV detection rates in samples collected 1982-89 and 1991-93



GW = Geriatric wards, OPH = Old peoples homes, HR = Hotels and restaurants, HWG = Hospital wards, general, SCH = Schools, SPC = Sporadic cases (not associated with outbreaks)

Figure 27. Distribution of PBVs detected in each age group for samples collected 1982-89 and 1991-93 (%)



C) Hospitalised patients with and without gastroenteritis as the main presenting illness

Seventy nine faecal samples were sent to VRD by Dr Hambling at Leeds PHL for PBV screening by PAGE. They were originally extracted by the phenol/chloroform method, but were subsequently retested using the 'Boom' extraction method.

Study population

The clinical diagnosis or main presenting illness were grouped into seven categories: Gastroenteritis (Gastro) which includes diarrhoea & vomiting (D & V), loose stools and enteritis; Neurological (Neuro) includes viral meningitis and meningitis; Lower Respiratory Tract Infections (LRTI) includes whooping cough, pneumonia, Legionnaires disease, and asthma; Upper Respiratory Tract Infections (URTI) includes tonsillitis; Rashes and Fevers (R & F) includes chickenpox, glandular fever, pyrexia of unknown origin (P.U.O.), and mumps; 'Others' includes post heart surgery, aplastic anaemia, acute myeloblastic leukaemia (A.M.L.), bone marrow transplant (B.M.T.), cerebrovascular accident (C.V.A.), supraventricular tachycardia (S.V.T.), haemolytic uremic syndrome (H.U.S.), jaundice and cystic fibrosis; and the last group is classed as Not Known (NK). The number of patients in each of the groups is shown as a percentage of the total (Figure 28.). The 79 patients were also further divided into two groups, those with gastroenteritis (30) and those without gastroenteritis (49).

The ages of the patients were grouped as follows: A -Infants (0 to 5 years) 16/79 (20%); B -Children (6 to 17 years) 29/79 (37%); C -

Adults (18 to 65 years) 19/79 (24%); D -Elderly (>65 years) 9/79 (11%) and E -Not known 6/79 (8%).

PBV detection

Three of 79 (4%) patient samples were positive for PBV using the phenol/chloroform (P/C) method for nucleic acid extraction (Figure 29.) and eleven samples (14%) were positive for PBV using the 'Boom' method for nucleic acid extraction. Several of these strains are shown in (Figures 30 and 31). Four of these samples 10, 13, 14, and 50 were very weak positives which were at the threshold of detection by PAGE and silver staining. Bands were visible on the gel when viewed on a light box, but were too faint to be photographed with the available equipment.

The prevalence of PBV in patients with gastroenteritis was 4/30 (13%) and in the patients without gastroenteritis was 7/49 (14%), indicating no obvious association with gastroenteritis. In order to investigate a possible association with other clinical syndromes, patients without gastroenteritis as the main presenting illness were examined for PBV. In these patients PBV were distributed as follows: P.U.O. (2) ; chickenpox (1); glandular fever (1); viral meningitis (1); tonsillitis (1); and mumps (1). When the illnesses were grouped into the seven categories the prevalence of PBV was 5/10 (50%) in the rashes and fevers group, 1/15 (7%) in the neurological group, and 1/2 (50%) in the upper respiratory tract infections group.

The distribution and prevalence of PBV by age of patients as a total group of hospitalised patients were as follows: Infants: 2/16 (13%); Children: 6/29 (20%); Adults: 1/19 (5%); Elderly: 1/9 (11%); and Not known 1/6 (16%) (Figure 32.). The distribution of patients, clinical diagnosis, age and PBV detection are shown in Table 13.

Table 13. **Hospitalised patients, clinical diagnosis, age and PBV detection**

Patient No.	Clinical diagnosis	Age (years)	PBV
1	NK	4	Neg
2	"	4	"
3	Vomiting	3	"
4	Diarrhoea	5	Pos
5	Vomiting	4	Neg
6	Gastroenteritis	6	"
7	Diarrhoea	29	"
8	P.U.O	9	Pos
9	Whooping cough	8	Neg
10	Chickenpox	6	Pos (weak)
11	Asthma, pneumonia	3	Neg
12	Post heart surgery	9	Neg
13	Glandular fever	14	Pos (weak)
14	Viral meningitis	8	Pos (weak)
15	Gastroenteritis	4	Neg
16	Viral meningitis	17	"
17	Aplastic anaemia	24	"
18	P.U.O, diarrhoea	42	"
19	Pneumonia, D & V	22	"
20	Legionnaires disease	12	"
21	NK	20	"
22	Diarrhoea & vomiting	NK	"
23	Gastroenteritis	4	"

Table 13. **Cont'd.**

Patient	Clinical diagnosis	Age (years)	PBV
24	Meningitis	15	"
25	Viral meningitis	15	"
26	Watery stools	4	Pos
27	Chickenpox	24	Neg
28	Jaundice	55	"
29	Tonsillitis	11	Pos
30	Viral meningitis	14	Neg
31	"	8	"
32	Asthma	13	"
33	Gastroenteritis	5	"
34	A.M.L., post B.M.T.	44	"
35	Gastroenteritis	3	Neg
36	Chickenpox	11	"
37	Tonsillitis	16	"
38	Bloody diarrhoea	8	"
39	Diarrhoea	9	"
40	Meningitis	31	"
41	C.V.A	77	"
42	Loose stools	82	"
43	Meningitis	10	"
44	NK	NK	"
45	Diarrhoea & vomiting	69	"
46	Loose stools	5	"
47	NK	NK	"
48	Glandular fever	NK	"

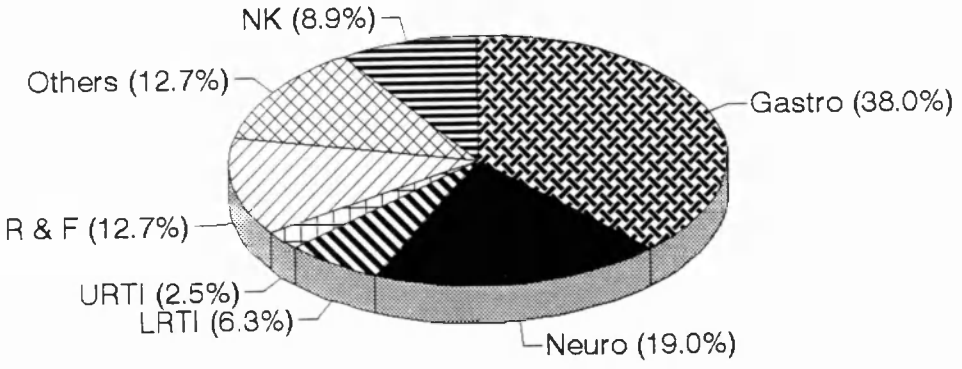
Table 13. Cont'd.

Patient	Clinical diagnosis	Age (years)	PBV
49	NK	36	"
50	Diarrhoea	62	Pos (weak)
51	"	NK	Neg
52	Diarrhoea & vomiting	77	"
53	"	16	"
54	P.U.O.	52	"
55	Enteritis	72	"
56	Viral meningitis	12	"
57	Diarrhoea	83	"
58	P.U.O.	NK	Pos
59	Diarrhoea & vomiting	45	Neg
60	Viral meningitis	14	"
61	Mumps	10	Pos
62	Diarrhoea & vomiting	71	Neg
63	Viral meningitis	13	"
64	Gastroenteritis	5	"
65	Glandular fever	22	"
66	Meningitis	3	"
67	NK	12	"
68	Gastroenteritis	20	"
69	Diarrhoea	4	"
70	"	35	"
71	Meningitis	8	"
72	Diarrhoea & vomiting	39	"
73	S.V.T.	9	"

Table 13. **Cont'd.**

Patient	Clinical diagnosis	Age (years)	PBVs
74	Enteritis	76	Pos
75	Cystic fibrosis	23	Neg
76	H.U.S.	3	"
77	Diarrhoea & vomiting	66	"
78	Meningitis	9	"
79	Enteritis	20	"

Figure 28. **Clinical diagnosis of hospitalised patients**



Patient numbers by clinical diagnosis

Gastro = Gastroenteritis (30); Neuro = Neurological (15); LRTI = Lower respiratory tract infection (5); URTI = Upper respiratory tract infection (2); R & F = Rashes and Fevers (10); Others (10); and NK = Not known (7).

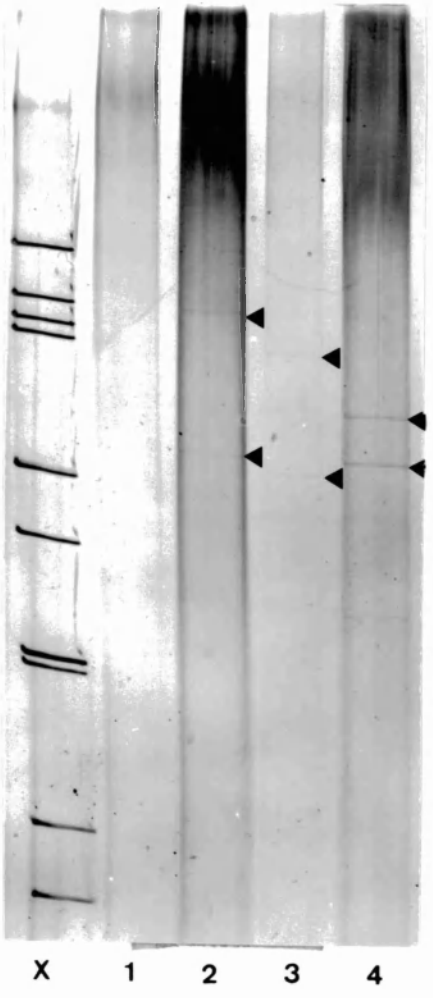


Figure 29. **PAGE showing three PBV strains detected in the hospitalised patients using the P/C method.**
Lane 1 negative, lane 2 sample 4, lane 3 sample 10, and lane 4 sample 26. Lane X is SA11 marker

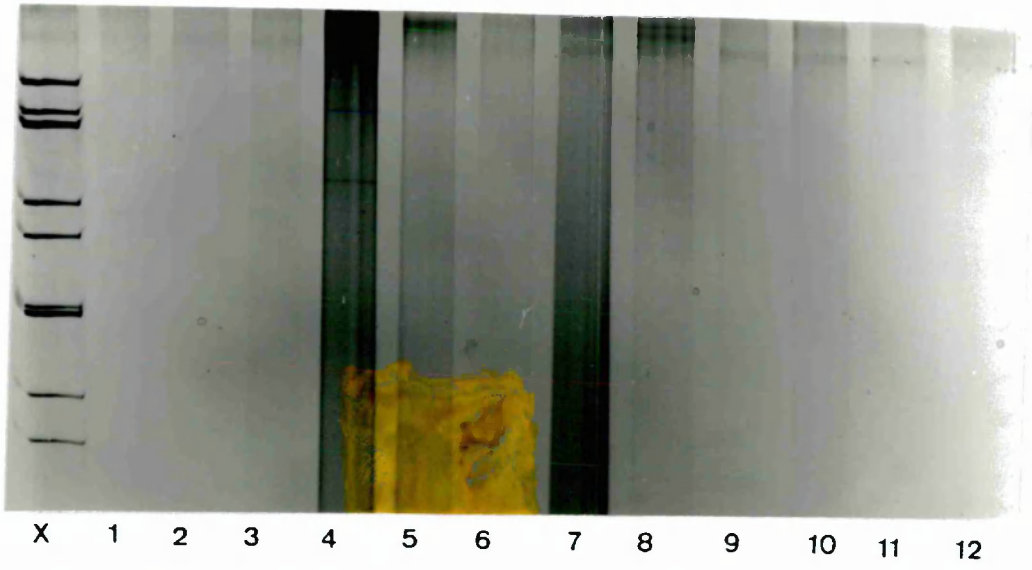


Figure 30. PAGE analysis of hospitalised patients- samples 1 to 12

Lane 1 to 12 are samples 1 to 12. Samples 4 , 8 and 10 (very weak) are positive for PBV. Lane X is SA11 marker

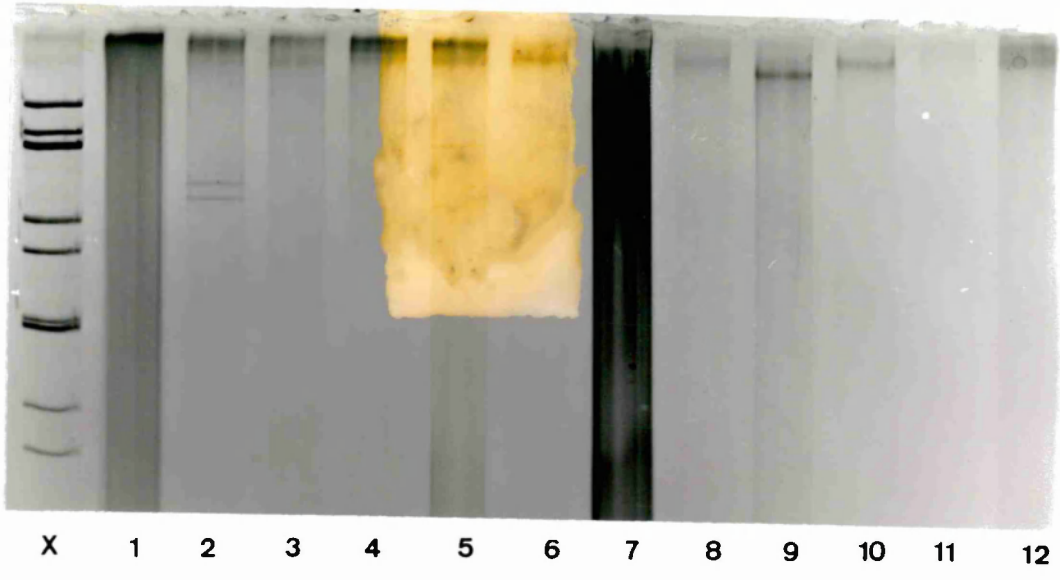
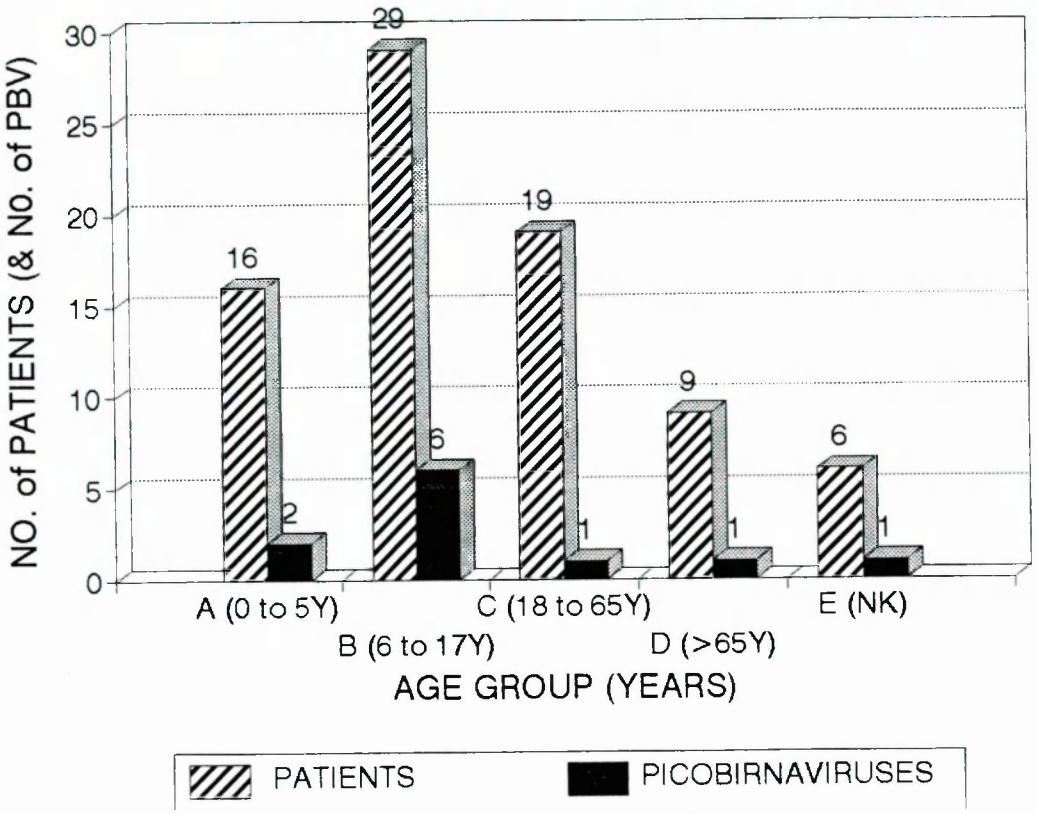


Figure 31. PAGE analysis of hospitalised patients- samples 25 to 36

Lane 1 to 12 are samples 25 to 36. Samples 26 and 29 are positive for PBV. Lane X is SA11 marker

Figure 32. Distribution of patients by age group and PBV in each age group



A = Infants; B = Children; C = Adults; D = Elderly; E = Not Known

D) **HIV-infected patients with and without diarrhoea**

This study was initiated to investigate the prevalence of picobirnaviruses in HIV-infected patients and to see if there was a link with PBV and diarrhoea. Faecal samples were collected from patients with and without diarrhoea, and were sent to VRD by clinicians in The Virology Department at The Royal Free Hospital. These samples were originally screened using the phenol/chloroform method of nucleic acid extraction and PAGE analysis. Using this technique two PBV (1.5%) were detected in 133 patients. The patient samples were later retested using the 'Boom' method of nucleic acid extraction and PAGE analysis and the detection of PBV described are based on this technique.

Study population

In all 133 patients were involved in the study and 237 samples collected, with multiple samples from some patients and single samples from others. The prevalence of PBV in HIV-infected patients was examined in two ways, firstly the 133 patients were tested (with the first sample collected as the test sample), and secondly the total number of patient samples (more than one sample from some patients) were analysed. The type of faecal sample were divided into three groups as follows: Diarrhoea, Non-Diarrhoea and Not Known.

PBV detection

Picobirnaviruses were detected by PAGE in 7/133 (5%) of HIV-infected patients. In the HIV-infected patient groups, PBV were distributed as follows: Diarrhoea: 3/112 (2.7%); No Diarrhoea: 3/11 (27%); Not Known: 1/10 (10%) (Figure 34.).

picobirnaviruses were detected in 8/237 (3.5%). In the breakdown of all of the samples by the HIV-infected group, PBV were distributed as follows: Diarrhoea: 3/170 (1.8%); No Diarrhoea: 4/24 (17%); and Not Known: 1/43 (2.3%) (Figure 35.).

The genomes of the PBV strains detected were examined (for genome sizes see Table 7.). From patient 13 seven samples were collected of which two were positive for PBV (strain P1 and P2), but the two strains were slightly different. The seven faecal samples collected from patient 13 are shown in Table 15., by date of collection, type of sample, and PBV detection. From patient 12 one faecal sample was obtained that contained two different strains of PBV (strains P8-1 and P8B). The sample from patient 58 gave an atypical profile that was not classed as a PBV strain (strain P5). Details of patients with PBV strains are given in Table 14. and genome profiles of the strains are shown in Figure 33. (same as Figure 7.). The strains P1, P2, P7, and P8-1 had similar genome profiles and strains P4, P6, and P8-2 had similar genome profiles; indicating two possible genomic groups.

There were seven distinct PBV strains detected in six patients (with the first PBV in the first sample counting as the strain type) and there were five strains in five individual patients (P1, P3, P4, P6 and P7, see Figure 33.) and one patient seemed to be infected with two strains (P8-1 and P8-2, see Figure 33.).

Table 14. **Picobirnavirus strains in HIV-infected patients**

Sample	Patient No.	Clinical presentation	PBV strain
1	13	Diarrhoeal	P1
2	13	Non-diarrhoeal	P2
3	29	Not known	P3
4	46	Diarrhoeal	P4
-	58	Diarrhoeal	P5 (atypical)
5	61	Non-diarrhoeal	P6
6	65	Diarrhoeal	P7
7	12	Non-diarrhoeal	P8-1
8		"	P8-2

Table 15. **Distribution by date of patient 13 faecal samples and the PBV detected in them**

Sample	Clinical presentation	Date	PBV
1	Diarrhoeal	17/3/92	+
2	Not Known	26/3/92	-
3	Diarrhoeal	NK	-
4	Non-diarrhoeal	30/3/92	+
5	Diarrhoeal	30/3/92	-
6	Diarrhoeal	31/3/92	-
7	Non-diarrhoeal	2/4/92	-

NK= Not known (although sample collected between 26/3/92 and 30/3/92)

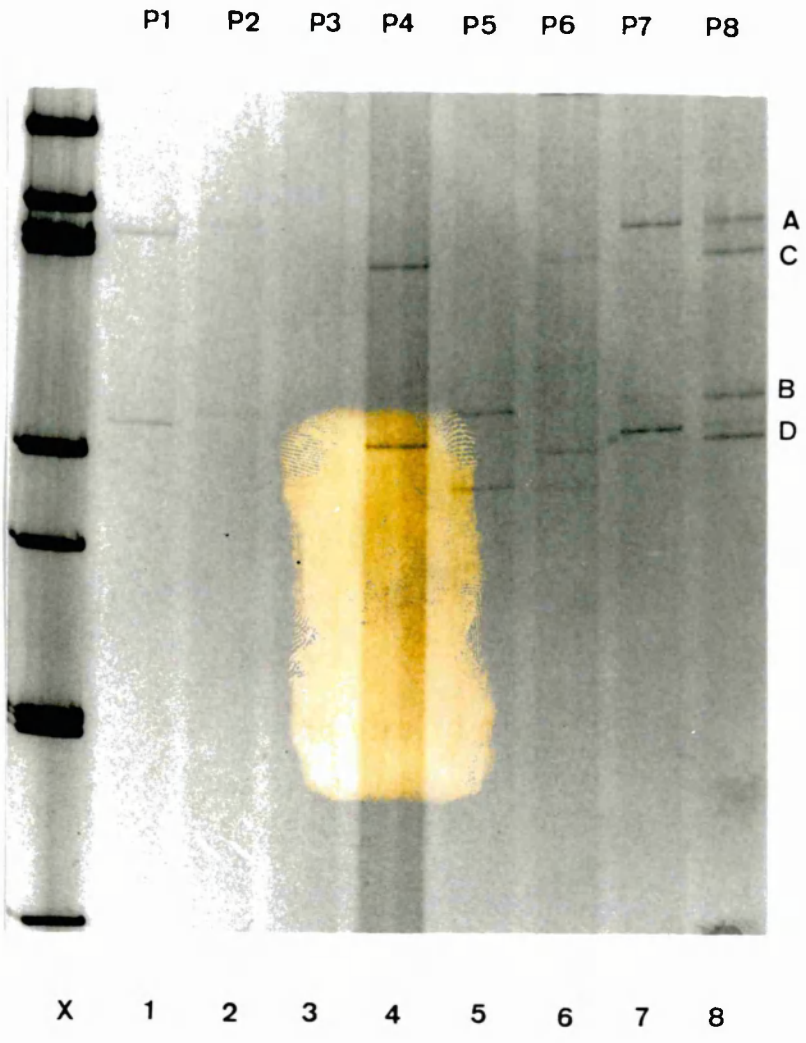


Figure 33. **PAGE analysis of PBV strains detected in HIV-infected patients**

Lane 1 to 8 show PBV strains P1, P2, P3, P4, P5 (atypical profile), P6, P7, P8-1 (A & B), and P8-2 (C & D) (see Table 14. for further details). Lane X is SA11 marker

Figure 34. Distribution and incidence for PBV in individual HIV-infected patients

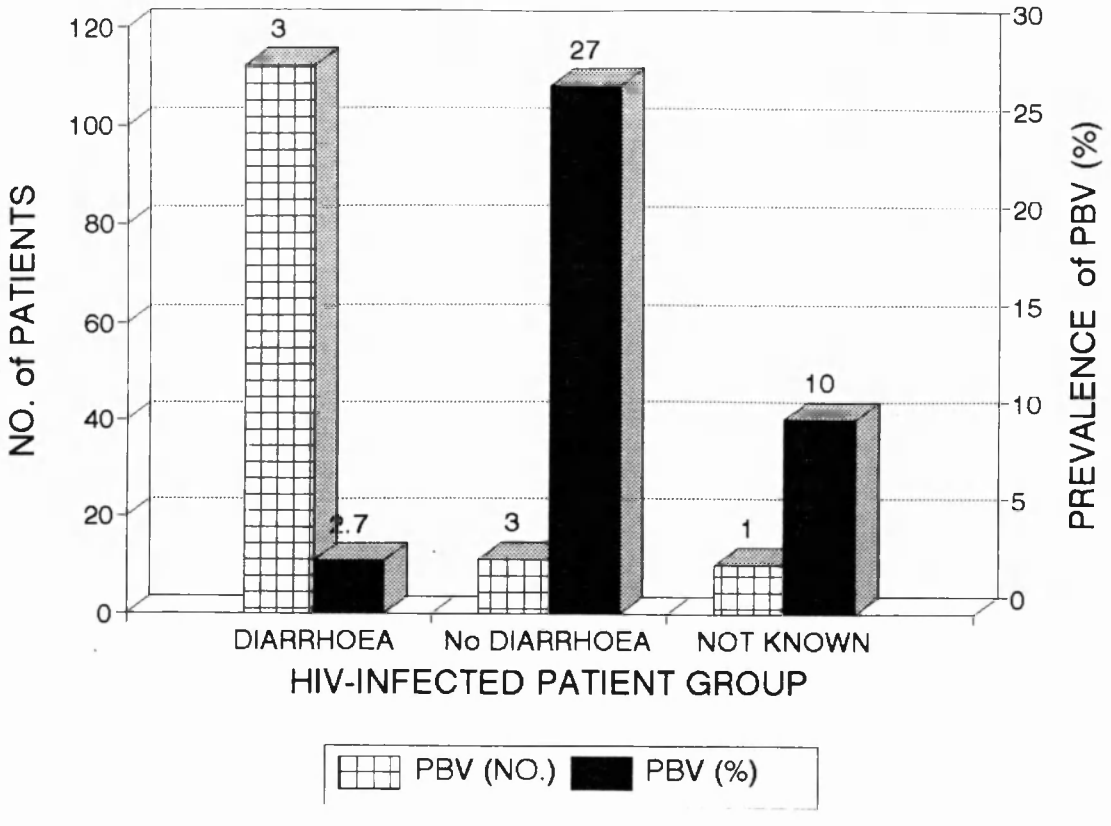
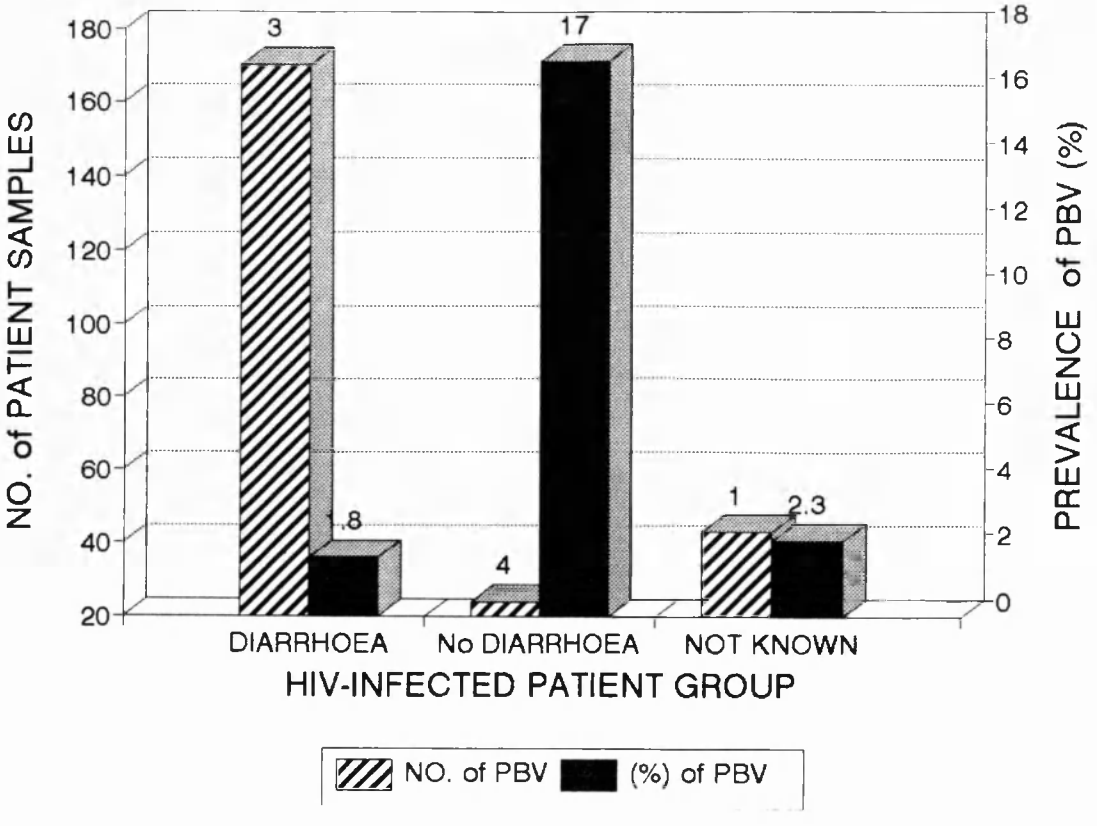


Figure 35. Distribution and prevalence for PBV in the total number HIV-infected patients samples according to the presence or absence of diarrhoea



E) Sporadic cases of gastroenteritis

i) Sporadic cases of diarrhoea (PHL, Leeds)

Sixty nine faecal samples from patients with diarrhoea (sporadic cases) referred for analysis by General Practioners to Leeds PHL were tested. Of these samples 55 were bacterial pathogen negative and 14 were bacterial pathogen positive. The bacterial pathogens detected were *Shigella sonnei (6)*, *Salmonella enteritidis (6)*, *Salmonella typhimurium (1)* and *Salmonella berti (1)*. The samples were extracted using the phenol/chloroform method and PBV were detected in 3/55 (5.5%) of the bacterial pathogen negative diarrhoeal samples, and in none of the bacterial pathogen positive samples. Overall, PBV were detected in 3/69 (4.3%) of sporadic cases of diarrhoea. No further details were available on age or clinical or virological diagnoses for these patients.

ii) Excretion of PBV in a sporadic case of gastroenteritis

A male staff member in his early thirties, presented with mild diarrhoea for one day in early June 1989 and he recovered fully. A faecal sample was shown to be PBV positive and a follow-up study of subsequent faecal samples was undertaken. The first six faecal samples were collected at approximately ten day intervals day 1 to day 57, a further six samples were collected from day 110 to day 138, and a final follow up sample was collected at day 288. PBV were detected by PAGE analysis (Figure 36.) in samples from day 1 to day 110, and were not detected in samples from day 120 to day 288 (Table 16.).

Table 16. **Detection of PBV in sequential faecal samples from an adult**

Sample No.	Day (post illness)	VRD No.	PBV
1	1	21975/89	Pos
2	15	22145/89	Pos
3	22	22940/89	Pos
4	32	24500/89	Pos (weak)
5	43	26455/89	Pos
6	57	28351/89	Pos
7	110	35693/89	Pos
8	120	36946/89	Neg
9	127	37721/89	Neg
10	128	37880/89	Neg
11	138	44425/89	Neg
12	288	11665/90	Neg

Pos = positive, Neg = negative

The PBV positives from day 1 to day 110 are illustrated in Figure 36.

Figure 36. **Sequential excretion of a PBV strain as analysed by PAGE**

Lane 1 to 7 are samples 1 to 7 and correspond to days 1, 15, 22, 32, 43, 57 and 110. Lane X is SA11 marker (see Table 16. for details).

SECTION 3 Rabbit picobirnavirus characterisation

Section 3 describes the detection of endogenous or co-incidental PBV in rabbits during attempts to develop an *in vivo* propagation system using an animal model by oral inoculation of various small animals, especially rabbits, with picobirnaviruses. Attempts at an *in vitro* tissue culture system were also investigated.
The characterisation of the genome of several rabbit picobirnaviruses and rabbit picobirnavirus-like particles are also reported.

R8) Preliminary screening of animal study faeces by PAGE analysis
  a) Study 1. Various small animals
  b) Study 2. Young adult rabbits
  c) Study 3. Newly weaned rabbits
  d) Study 4. Re-inoculation of rabbits

R9) Sizing of picobirnavirus genomic segments detected in rabbit faeces

R10) Demonstration of the nature of picobirnavirus nucleic acid from rabbit faeces

R11) Determination of the buoyant density in CsCl of picobirnavirus particles from rabbit faeces

R12) Electron microscopy of picobirnavirus-like particles from CsCl gradient fractions

R13) Immune electron microscopy of picobirnavirus-like particles from rabbit faeces

R14) Tissue culture studies of picobirnaviruses

R8) Preliminary screening of animal faeces by PAGE

All faecal samples in the preliminary screening were extracted using the phenol/chloroform method.

A) Study 1. Various small animals

The small animals were inoculated with two PBV strains from human faecal samples (39177/89 and 21975/89) and a control.

Faecal samples were collected from the five different small animal species: rabbit (*Oryctolagus cuniculus*), rat (*Rattus norvegicus*), cavie (*Cavia porcellus*), hamster (*Mesocricetus auratus*), mouse (*Mus domesticus domesticus*) They were screened for picobirnavirus genomic bands by extraction of nucleic acid and PAGE analysis . The first faecal samples screened were from hamsters 3 & 4 (combined faeces from one cage), and 5 & 6; they were chosen because the faecal samples were moist, lacked form and the majority of faeces from all the other animals were solid and dry. The gel analysis revealed the presence of numerous bands (Figure 37.), which proved difficult to interpret and these specimens were stored for future reference.

Faecal samples were collected daily from each of the animals for 30 days following inoculation. PAGE was used to analyse faecal samples from days 0, 3, 6, 9, and 12 for initial screening from rabbits 5 (PBV 21975/89), 2 (control), rats 1 & 2 (PBV 21975/89) and rats 5 & 6 (control) (Figure 38.). PAGE analysis of these faecal samples did not produce the multiple bands as seen with the hamster faeces (Figure 37.). However two equimolar bands were detected in day 9 from rabbit 5 and were the first picobirnavirus bands observed in this group of small animals. The preliminary screening was also performed on cavie

3 & 4, cavie 5 & 6, mouse 3 & 4 and mouse 5 & 6 faecal samples (Table 4.), which all proved to be negative for PBV bands.

Following the detection of two equimolar bands in rabbit 5 day 9 faeces only, but not in day 6 or day 12, samples from days 3 to 14 were analysed (Figure 39.). The gel revealed that two equimolar bands were present in days 8, 9 and 10, with a peak by staining intensity in day 9. All 30 faecal samples from day 0 to day 29 for rabbit 5 were then analysed and PBV bands were not detected in any other days. The other rabbit (R6) that was inoculated with PBV strain 21975/89 was screened and no PBV bands were seen. The remaining animals were also screened and rabbit 1 and rat 3 & 4, mouse 1 & 2 and hamster 1 & 2, rabbit 3, rabbit 4 and cavie 1 & 2 (Table 4.) were negative for PBV bands. The faecal samples from rabbit 5 day 8 (R5-8), 9, 10 were stored at $4^{o}C$, and $-70^{o}C$ for further analysis.

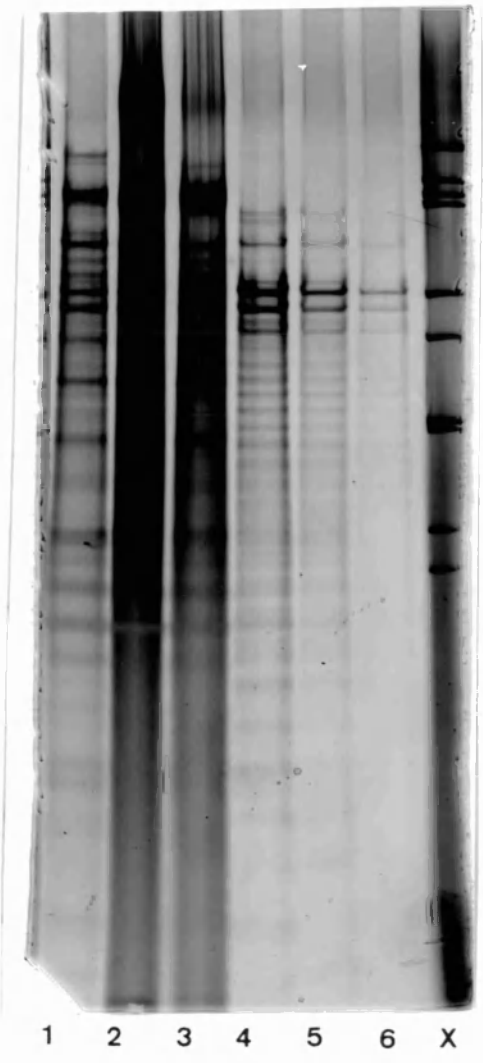


Figure 37. **PAGE analysis of hamsters 3 & 4, and 5 & 6 faecal samples**

Lane 1, 2 and 3 are day 3, 6 and 8 from hamster 3 & 4, and lane 4, 5 and 6 are same days for hamster 5 & Lane X is SA11 marker. Multiple bands present in lanes 1 to 6.

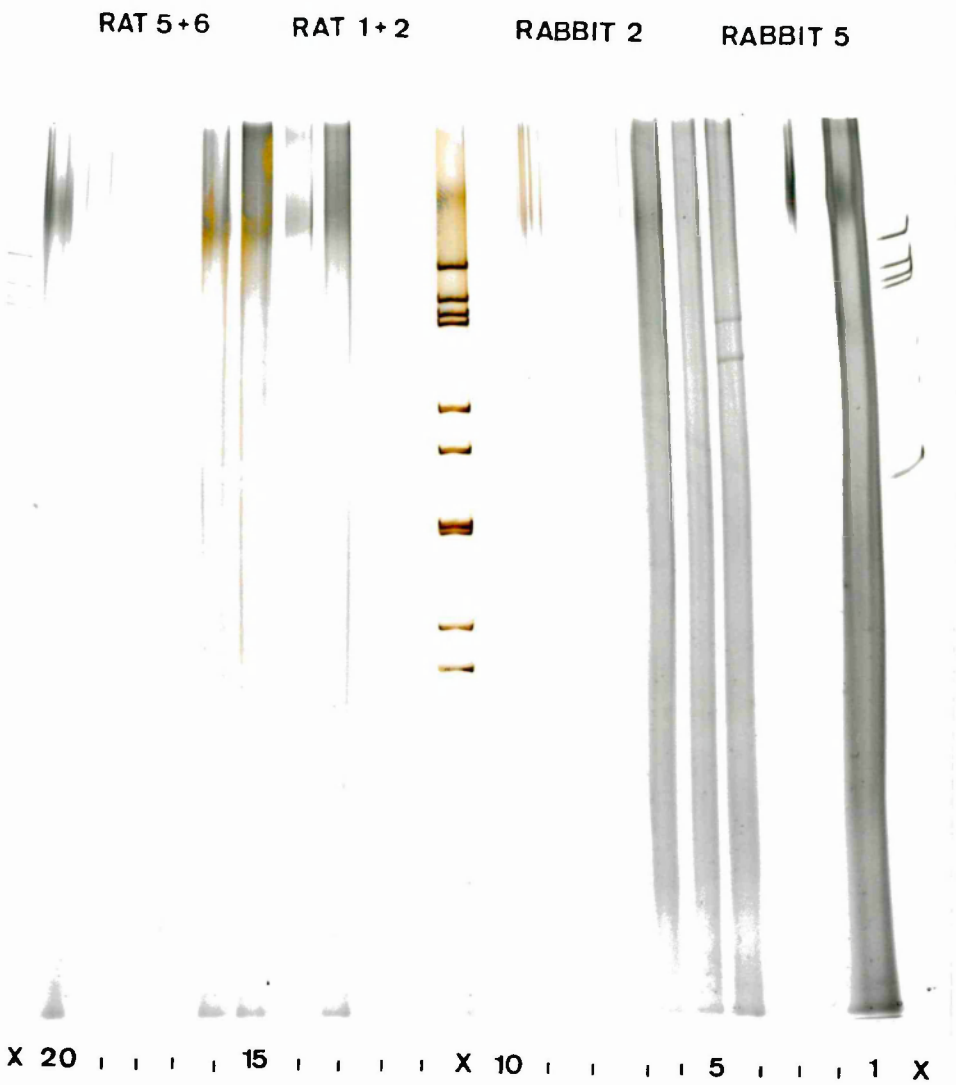


Figure 38. **PAGE analysis of day 0 to 12 faecal samples for rabbits 5 and 2, rat 1 & 2 and rat 5 & 6**

Lane 1 to 5 are day 0, 3, 6, 9, 12 for rabbit 5, lane 6 to 10 are the same days for rabbit 2, lane 11 to 15 same for rat 1 & 2, and lane 16 to 20 same for rat 5 & 6. Lane X is SA11 marker.

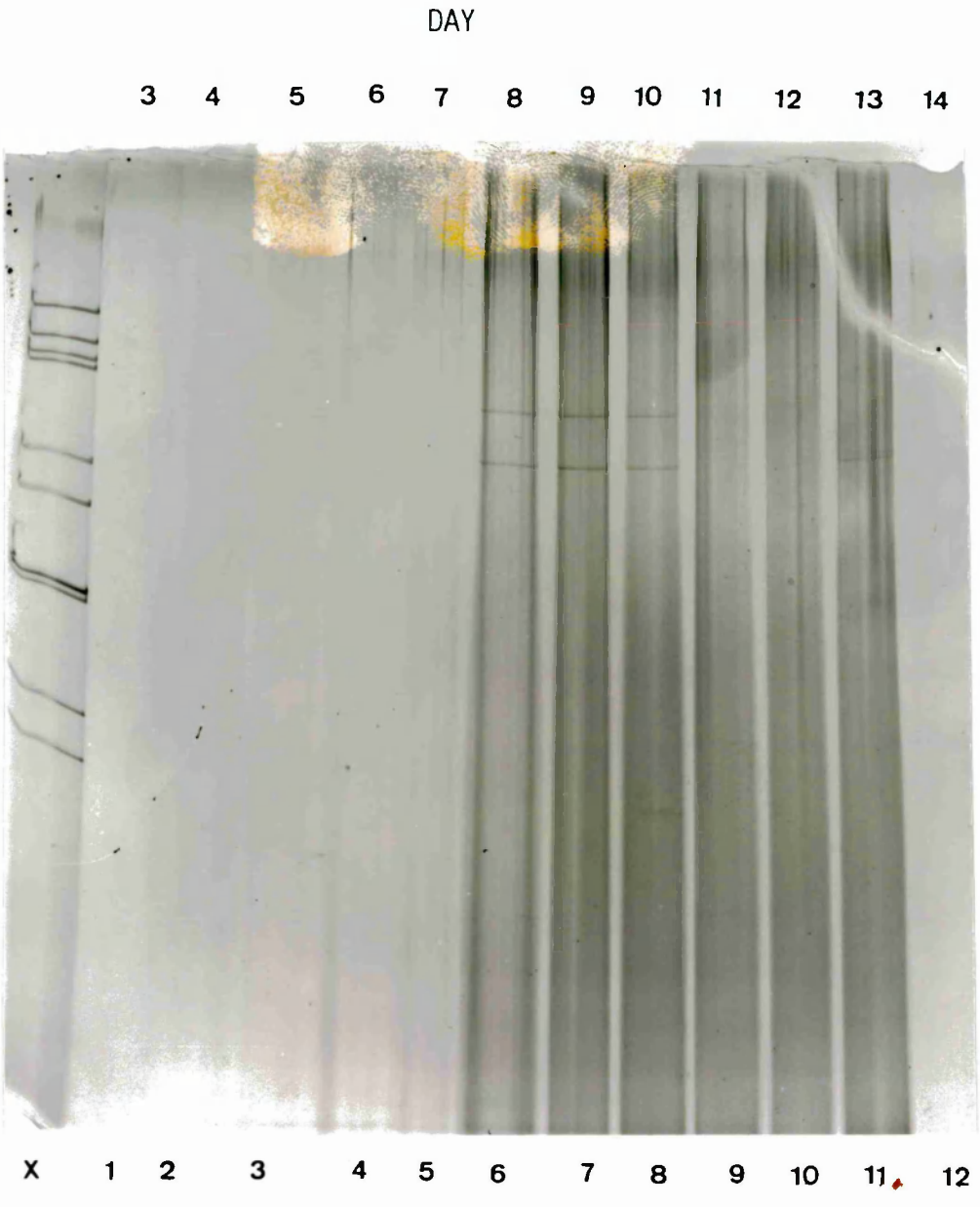


Figure 39. **PAGE analysis of rabbit 5 day 3 to day 14 faecal samples**

Lane 1 to 12 are day 3 to 14 and lane X is SA11 marker. PBV bands in lanes 6 to 8

B) Study 2. Young adult rabbits

These rabbits were inoculated with human PBV strain 21975/89 and rabbit PBV strain R5-9 and a control. Day 0 to day 15 faecal samples from rabbits R13 to R20 were screened as described in study 1. No picobirnavirus bands were detected in any of the specimens.

C) Study 3. Newly weaned rabbits

The 10 newly weaned rabbits R1 to R11 (no rabbit 8) were inoculated with the picobirnavirus strain detected in rabbit 5 from study 1 coded as R5-9. The distribution of PBV strains in the rabbits are shown in Table 19.

In rabbit 1, various bands were seen in days 0, 4, 5 and 9 (data not shown); however these bands appeared to be artefacts and were not present when the samples were re-extracted and re-run on a PAGE gel. Rabbit 2 showed no evidence of picobirnavirus bands being present in its faeces. In rabbit 3, faint bands were visible in days 1 and 2 (data not shown); however the bands were not uniform or equimolar and were not regarded as PBV bands. The specimens from rabbit 4 were negative up to day 14, this specimen had two faint PBV bands in day 14 (data not shown). In rabbit 5, PBV bands were demonstrated in day 0 to day 4 and very faint bands were seen in day 6 and 7 (Figure 40.). PBV bands were detected in rabbit 6 faecal samples from day 4 to day 14 (Figure 41.). Further samples from this rabbit were examined from day 42 to day 48, which were found to be positive (data not shown). Samples collected after day 48 were negative. In rabbit 7 PBV bands were demonstrated in samples collected on day 1 and day 2

and further bands were also detected in day 5 to day 11 (Figure 42.). The bands in the first two days samples were of a different profile to the bands detected in the subsequent samples. Rabbit 9 faecal samples were negative for PBV bands. In rabbit 10 PBV bands were clearly visible (Figure 43.). In samples collected on day 7 and 8 faint bands can be seen, with an increasing intensity (by silver staining) from day 9 to day 12 (peak day) and a fading of intensity from day 13. Rabbit 11 faecal samples were negative from day 0 to day 13, with two PBV bands appearing in day 14 sample (Figure 44.). Rabbits 3, 7 and 9 died during the course of the study, on days 7, 14 and 4 respectively. Faecal samples were taken from these rabbits post mortem and were negative by PAGE.

Figure 40. PAGE analysis of rabbit 5 day 0 to 14 faecal samples Lane 1 to 15 are days 0 to 14. Lane X is SA11 marker. PBV bands in lanes 1 to 5 and faint bands in lanes 7 and 8.

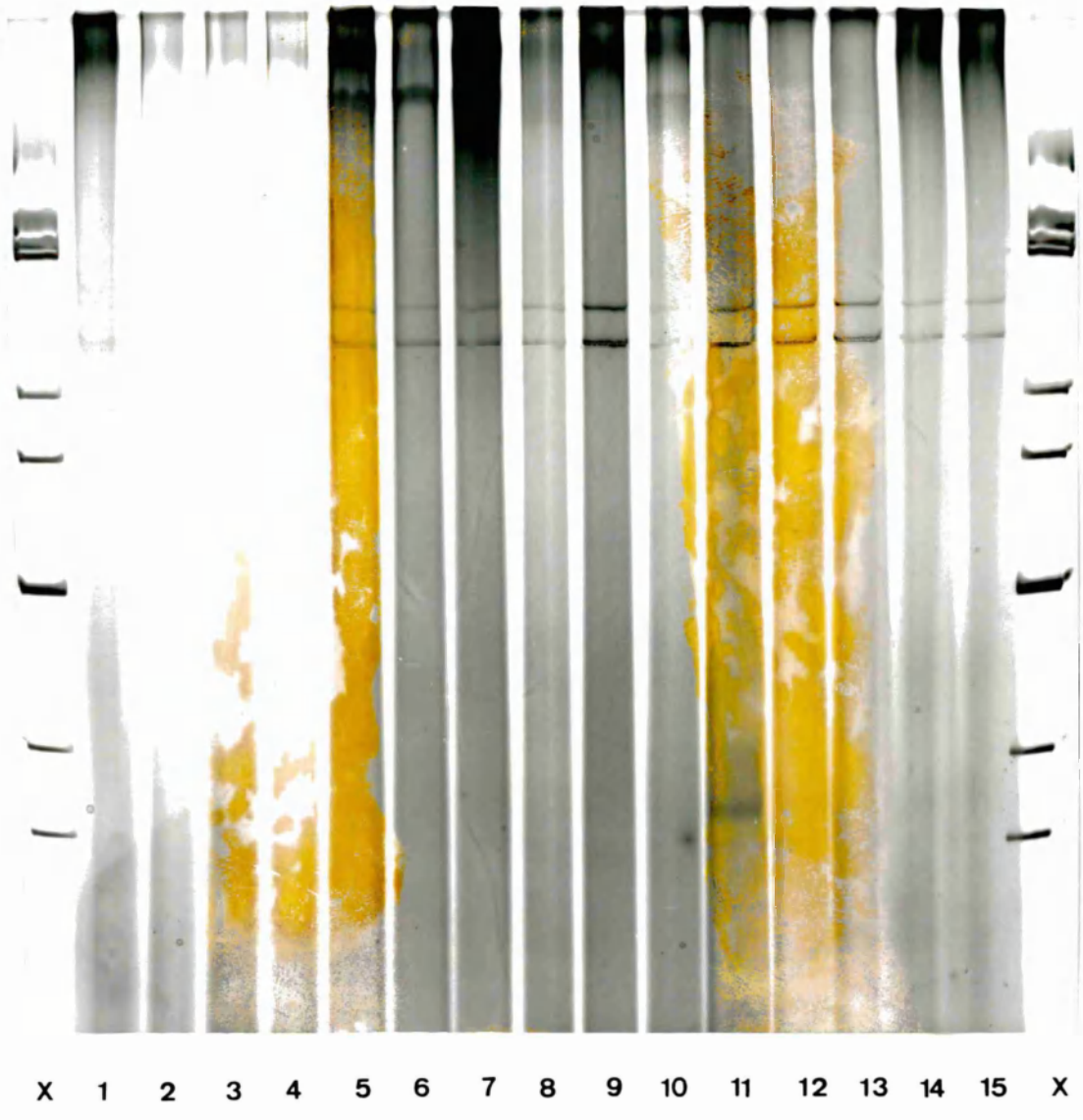


Figure 41. PAGE analysis of rabbit 6 day 0 to 14 faecal samples
Lane 1 to 15 are days 0 to 14. Lane X is SA11 marker.
PBV bands in lanes 5 to 15.

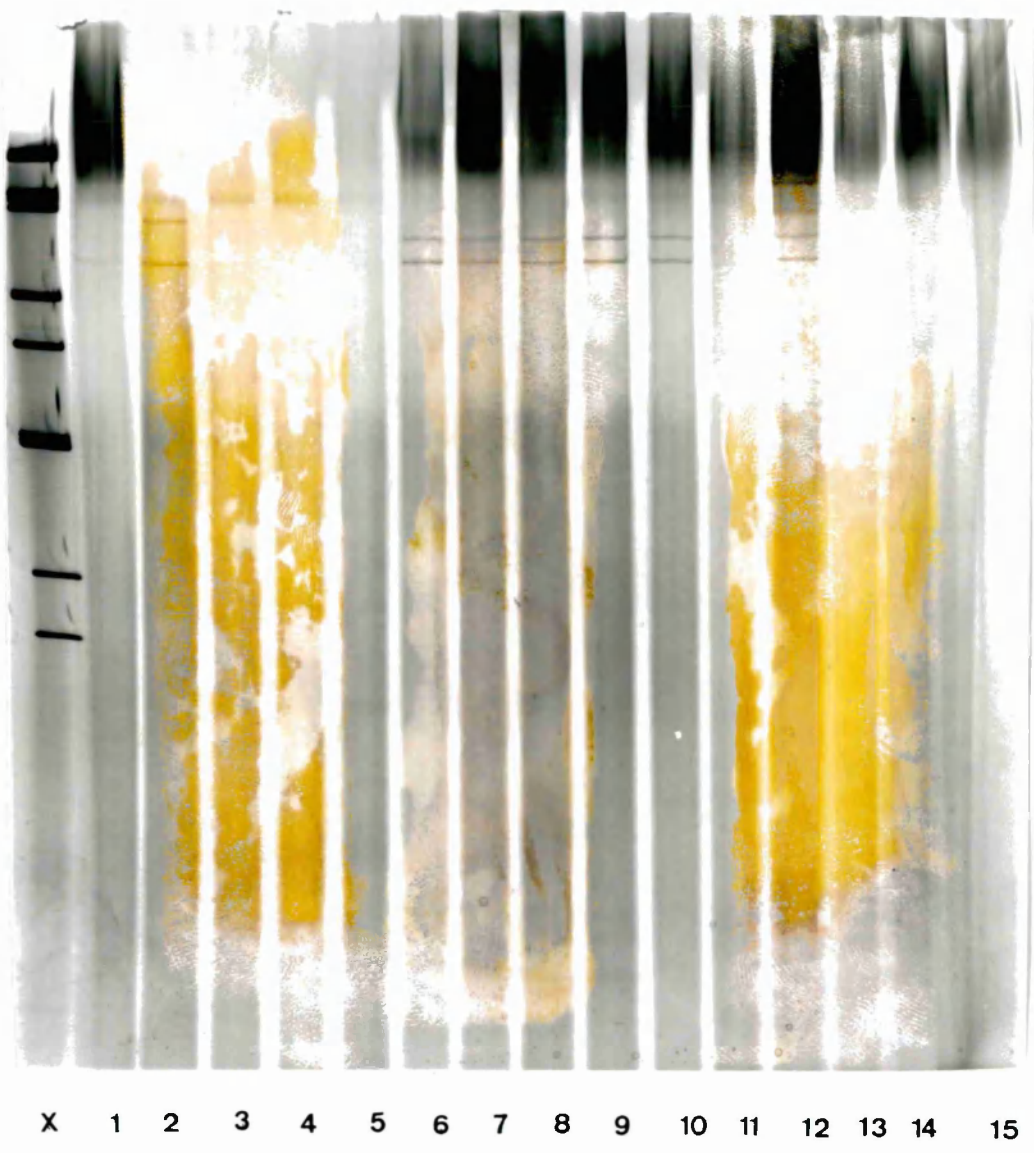


Figure 42. PAGE analysis of rabbit 7 day 0 to 14 faecal samples
Lane 1 to 15 are days 0 to 14. Lane X is SA11 marker.
PBV bands with an intermediate pattern in lanes 2 and
3, and PBV bands with a narrow pattern in lanes 6 to 12.

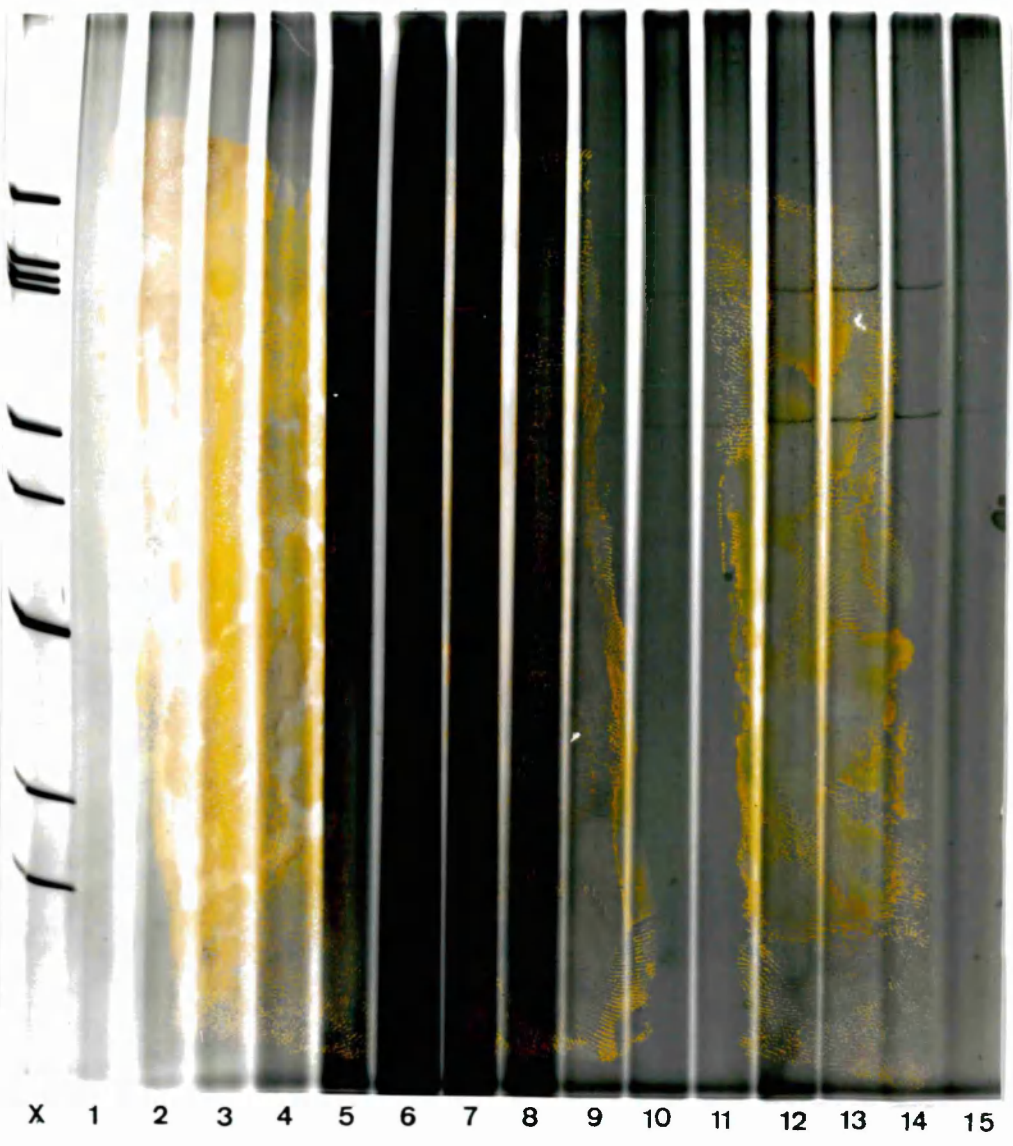


Figure 43. PAGE analysis of rabbit 10 day 0 to 14 faecal samples
Lane 1 to 15 are days 0 to 14. Lane X is SA11 marker.
PBV bands with a wide pattern in lanes 8 to 15.

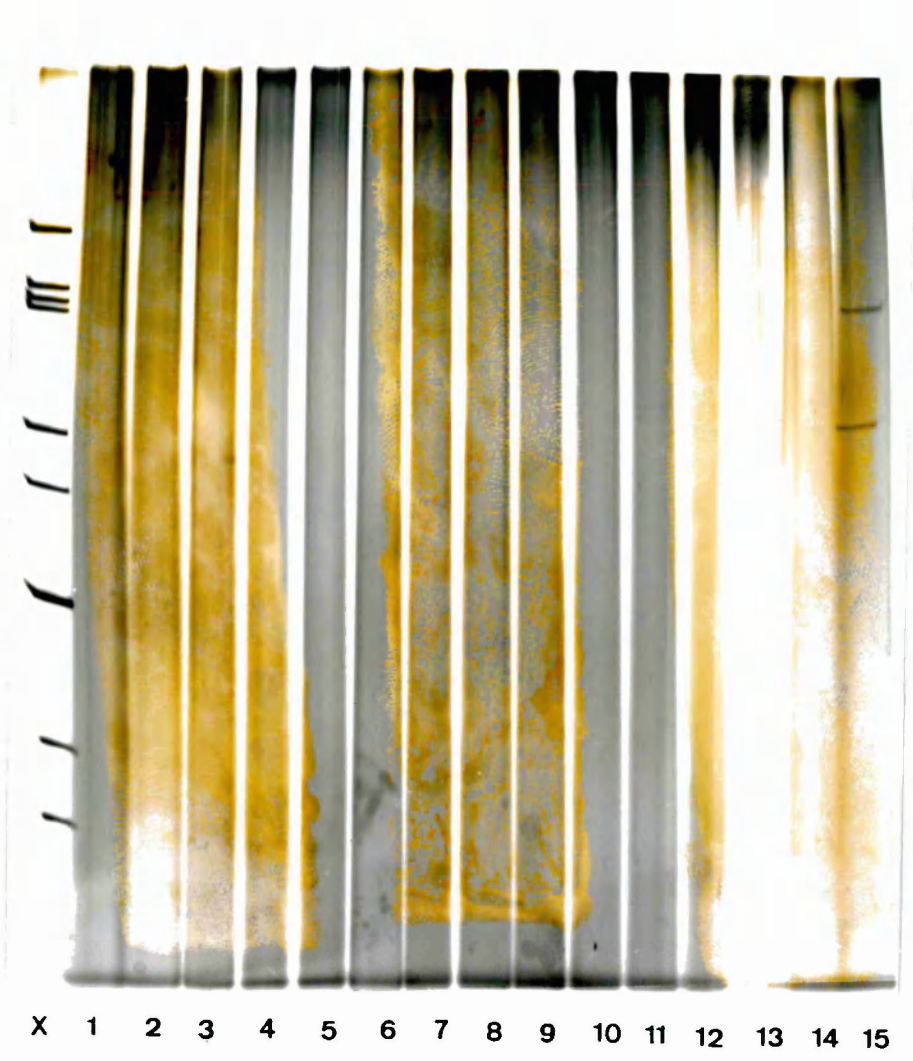


Figure 44. PAGE analysis of rabbit 11 day 0 to 14 faecal samples
Lane 1 to 15 are days 0 to 14. Lane X is SA11 marker.
PBV bands with wide pattern in lane 15 only.

D) Study 4. Re-inoculation of rabbits

There were 9 rabbits examined in this study, rabbits R17 and R20 from study 2 and rabbits R1, R2, R3, R4, R5, R6, R10 and R11 (study 3); all were re-inoculated with RPBV strain R5-9.

The faeces from the rabbits in this study were screened using the same methods as for the other animal studies. Samples from only two rabbits (rabbits 17 and 10) were positive. For rabbit 17, two bands representative of picobirnaviruses can be seen in day 9, with stronger bands on day 10 (Figure 45.). Faecal samples collected on day 14 were also positive, but the bands were weak (data not shown). The sample collected on day 15 was negative. PBV bands were detected in samples from only one other rabbit (rabbit 10) in this study. Bands were detected in the day 5 sample, with weak bands in the day 6 sample (Figure 46.).

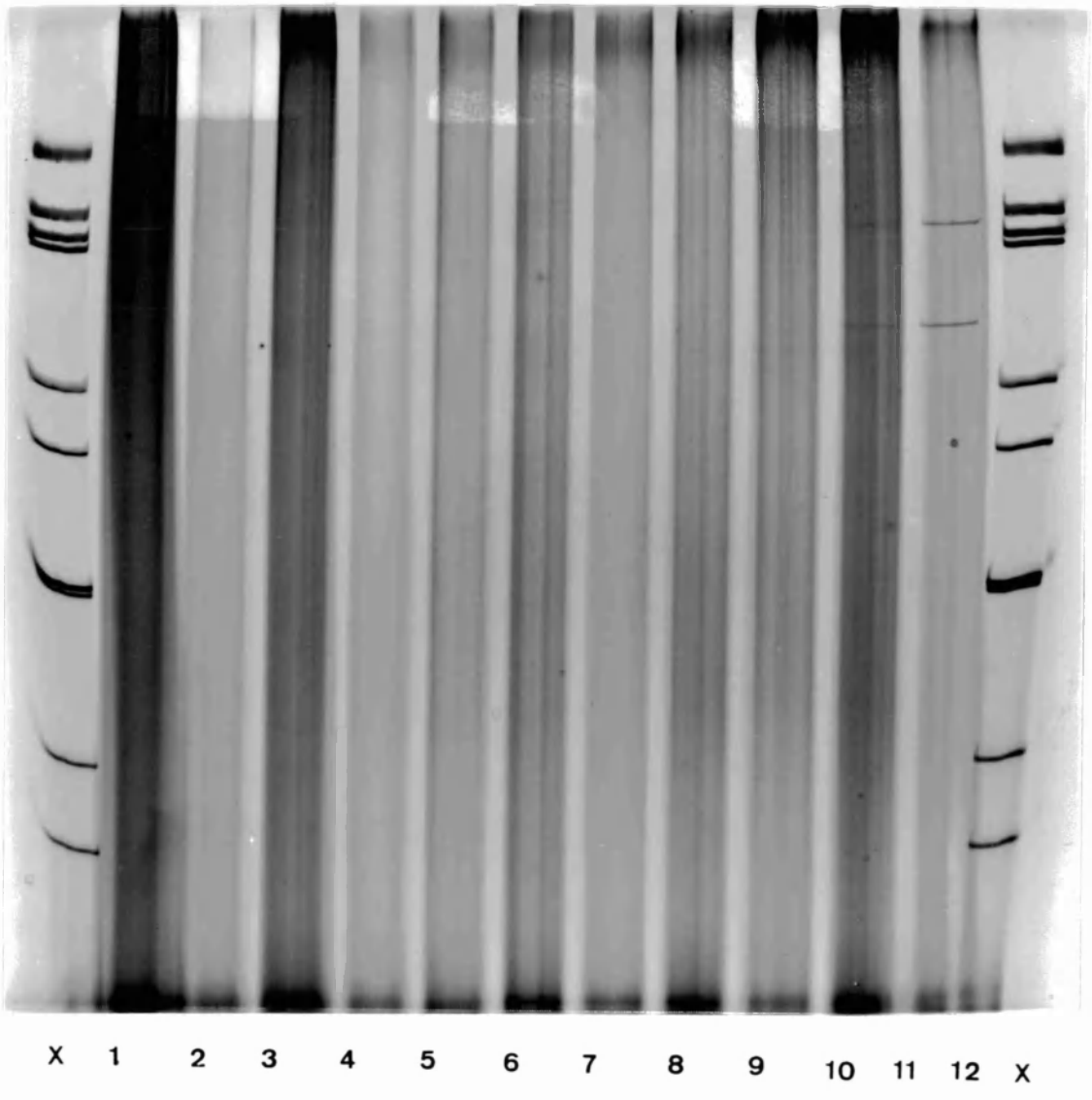


Figure 45. **PAGE analysis of rabbit 17 day 0 to 10 faecal samples (from study 4)**

Lane 1 to 12 are days 0 to 10. Lane X is SA11 marker. PBV bands with a wide pattern in lanes 11 to 12.

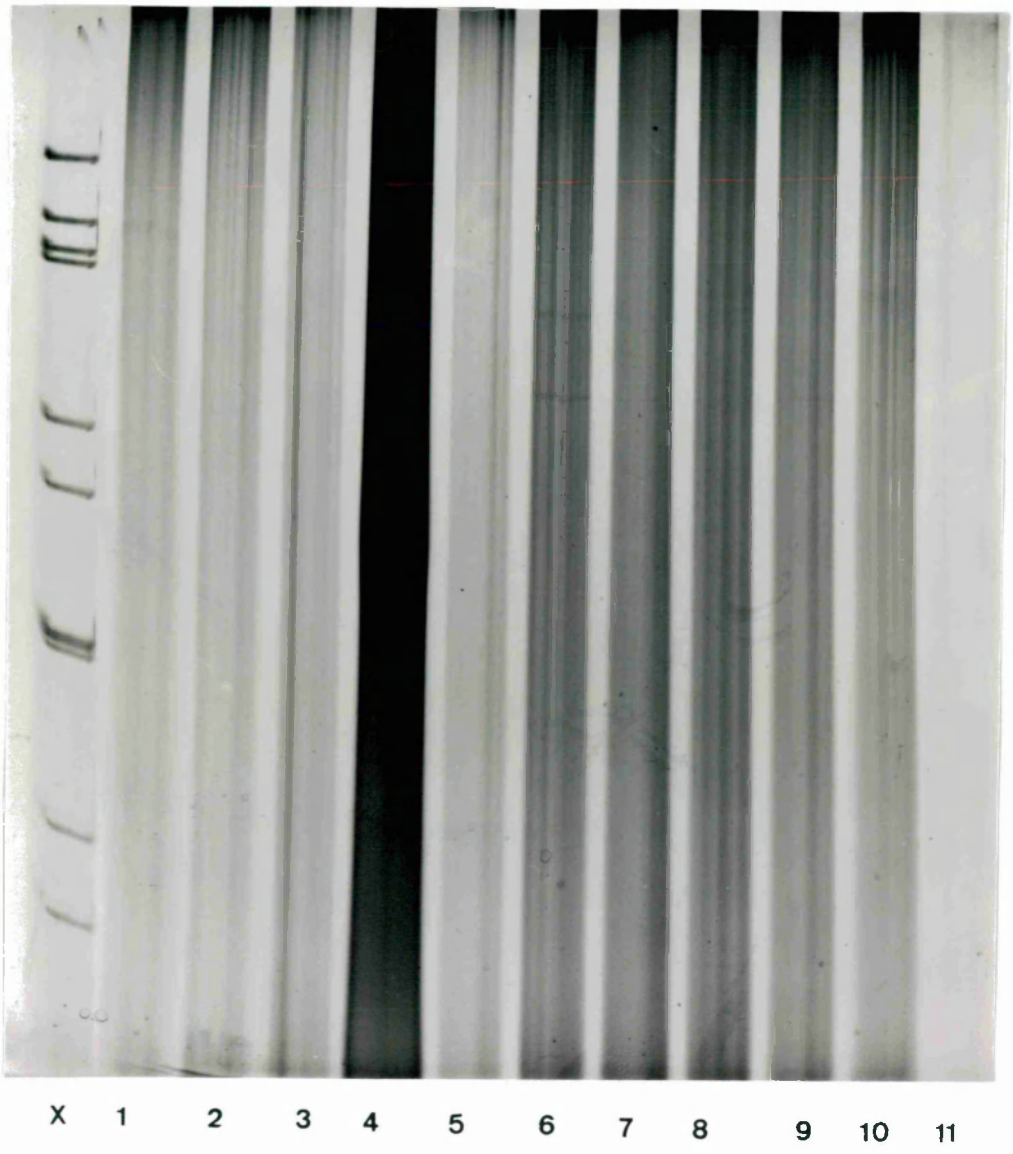
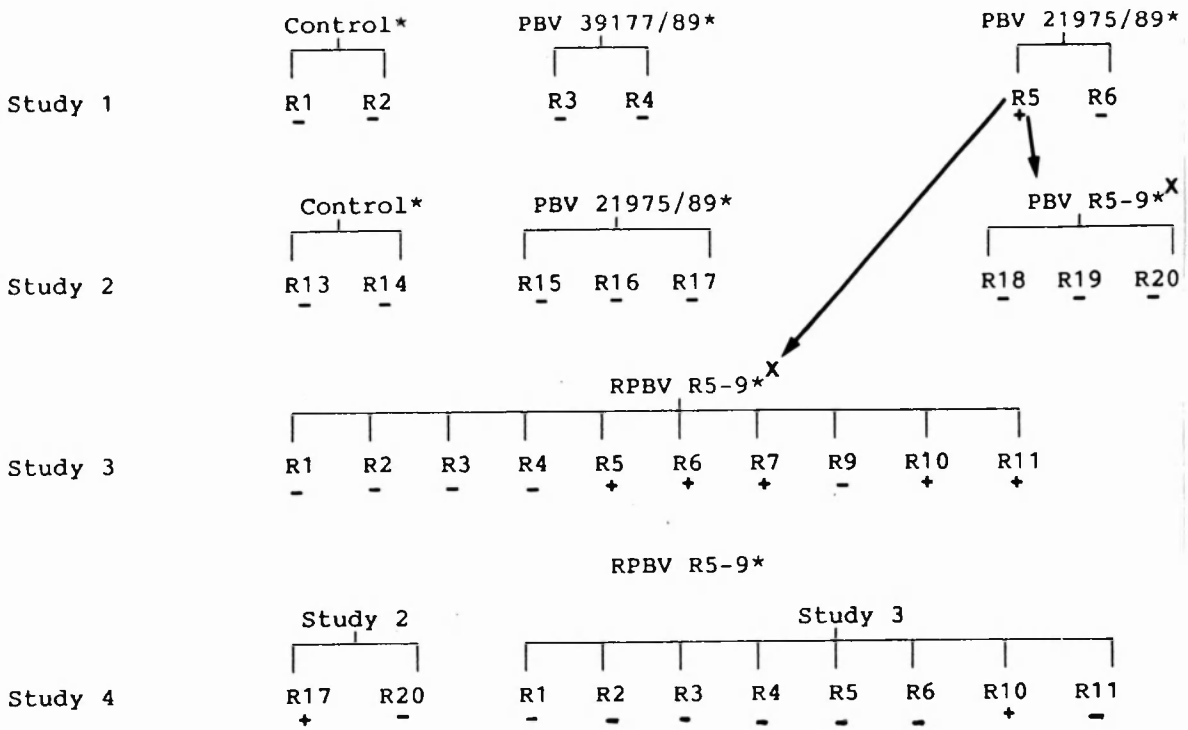


Figure 46. **PAGE analysis of rabbit 10 day 0 to 10 faecal samples**
Lane 1 to 11 are days 0 to 10. Lane X is SA11 marker.
PBV bands with an intermediate pattern in lane 6 and 7.

In total 10 rabbit picobirnavirus (RPBV) strains were detected during the animal studies and these are described in Table 19.

The relationships between the rabbits in the studies and the inocula used and the picobirnaviruses detected in them are expressed in Table 17. The genome patterns detected were assigned to three types; narrow, intermediate, and wide (Figure 48.).

Table 17. Relationships of the rabbits and inocula in the four studies



* = Inoculum + = PBV

R = Rabbit - = No PBV

x = R5 produced PBV strain R5-9

Study 1 = Young adult rabbits

Study 2 = Young adult rabbits

Study 3 = Newly weaned rabbits

Study 4 = Boosting of study 2 and 3 rabbits

R9) **Sizing of picobirnavirus genomic segments detected in rabbits**

The first picobirnavirus detected in rabbits was found in samples from rabbit 5, study 1 (Figure 39.). This strain was used for the majority of the characterisation studies of rabbit picobirnaviruses. The rabbit 5 strain was referred to as R5- day 9 (R5-9) or RE strain. The size of R5-9 genomic segments (Figure 47.) were calculated using the same procedure as for the human strains (see R2).

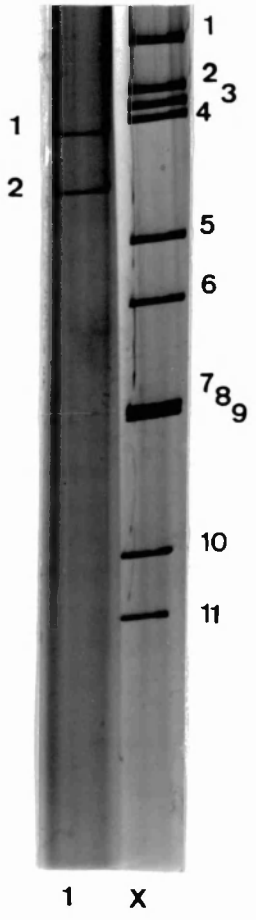


Figure 47. **Sizing of R5-9 picobirnavirus genomic segments by PAGE analysis.**

Lane 1 shows rabbit 5 - day 9 (R5-9) PBV strain, and lane X is SA11 marker.

The genomic segments of R5-9 and three further strains of picobirnavirus detected in rabbits (from study 3) were sized. The rabbit PBV strains are rabbit 6-day 9 (R6-9), rabbit 7-day 1 (R7-1), and rabbit 10-day 11 (R10-11). The PAGE gel is shown in Figure 48., and the genome size estimation is demonstrated in Table 18.

Table 18. Rabbit PBV genomic segments and estimation of segment sizes.

PBV strain	Seg. No.	PBV segment size base pairs(bp)	Total segment size (bp)	Strain type
R5-9	1	2300	4250	Intermediate
	2	1950		
R6-9	1	2140	4070	Narrow
	2	1930		
R7-1	1	2290	4220	Intermediate
	2	1930		
R10-11	1	2630	4470	Wide
	2	1840		

(data from Figure 47. and 48.)

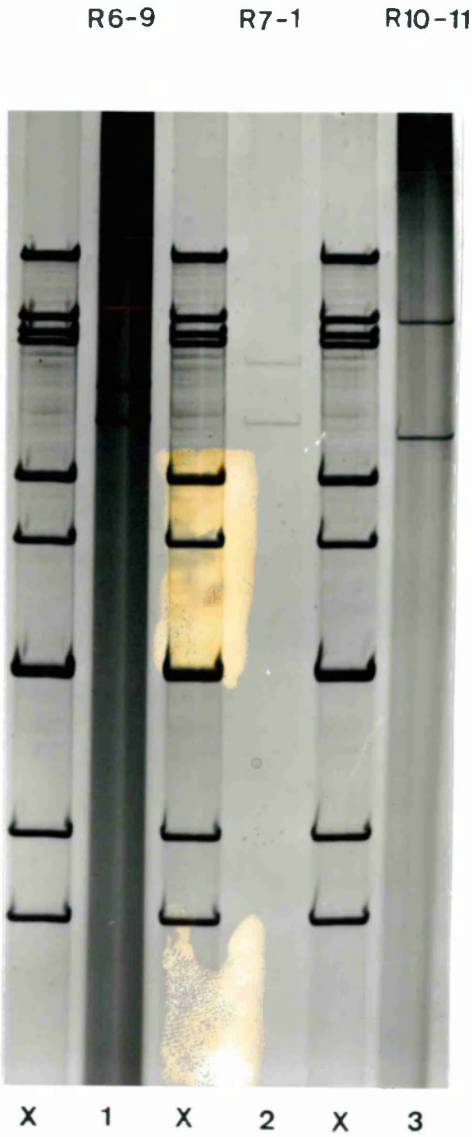


Figure 48. **Sizing of the genomic segments of three PBV strains detected rabbits in study 3 by PAGE analysis**

Lane 1 shows PBV strain R6-9 (narrow pattern), lane 2 R7-1 (intermediate pattern), and lane 3 R10-11 (wide pattern). Lane X is SA11 marker.

Table 19. Distribution of PBV strains in rabbits by day of collection and genome profile

		Faecal sample (day)														
Study No.	**Rabbit**	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	R5	-	-	-	-	-	-	-	I	I	I	I	-	-	-	-
3	R4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N
	R5	W	W	W	W	W	-	-	-	-	-	-	-	-	-	-
	R6	-	-	-	-	N	N	N	N	N	N	N	N	N	N	N+
	R7	-	I	I	-	-	N	N	N	N	N	N	N	-	-	-
	R10	-	-	-	-	-	-	-	W	W	W	W	W	W	W	W
	R11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	W
4	R17	-	-	-	-	-	-	-	-	-	W	W	W	W	W	W
	R10	-	-	-	-	-	I	I	-	-	-	-	-	-	-	-

PBV genome profiles (I= Intermediate, N= Narrow, W= Wide)

The distribution of PBV strains by genome profile in the rabbits in the three studies are shown in Table 19.

The narrow genome profile was represented by the three strains R4-14, R6-9, and R7-8. The segment 1 measured 2150 and segment 2 1930 base pairs. The intermediate genome profile was represented by R5-9, R7-1, and R10-5 and ranged from 2300 to 1800 base pairs. The wide genome profile was represented by R5-0, R10-11, R11-14, and R17-10 and ranged from 2630 to 1840 base pairs (Table 19.). The three narrow and three wide PBV genomic profiles from study 3 were co-electrophoresed (Figure 49.). There was no evidence that PBV had passaged in any of the rabbits inoculated by comparison of the genome profile of the inoculum and post-inoculum strains.

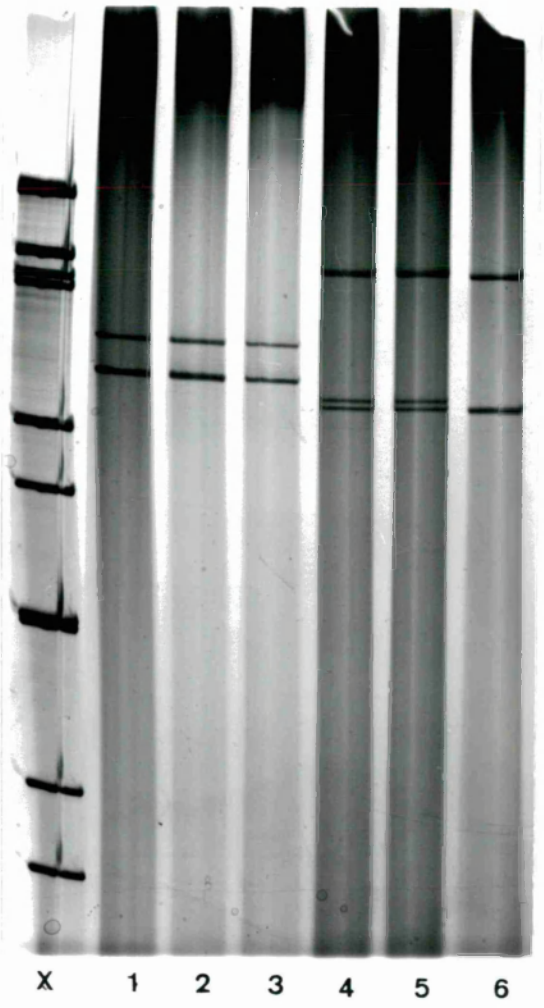


Figure 49. PAGE showing comparative genome profiles by co-electrophoresis of narrow and wide PBV strains from study 3.

Lane 1 shows rabbit 4-day 14 (R4-14) strain + R6-9, lane 2 R6-9 + R7-8, lane 3 R4-14 + R7-8, lane 4 R5-0 R10-11, lane 5 R10-11 + R11-14, lane 6 R5-0 + R11-14. Lane X is SA11 marker.

R10) Demonstration of the nature of picobirnavirus nucleic acid from rabbit faeces

The characterisation of the RPBV nucleic acid represented by two equimolar bands in rabbit 5 day 9 (R5-9) was performed as described in MM5. The results were essentially the same as those described for the PBV strains from human faecal samples (results section R3). A PAGE gel demonstrating the effect of different nucleases on rabbit PBV strain R5-9 nucleic acid and control nucleic acid are shown in Figure 50. and Table 20. The RPBV (R5-9) nucleic acid had the same digestion profile as rotavirus SA11 (dsRNA). and it was concluded that the two segments of RPBV (R5-9) were dsRNA.

Table 20. **Rabbit picobirnavirus (RPBV) strain R5-9 nucleic acid digestion data**

Lane		Nucleic acid	Nuclease	Result
	1	R5-9	No nuclease	No digestion
	2	"	RNase A	Digestion
A	3	"	RNase T1	No digestion
	4	"	RQ1 DNase	No digestion
	5	SA11 (dsRNA)	No nuclease	No digestion
	6	"	RNase A	Digestion
B	7	"	RNase T1	No digestion
	8	"	RQ1 DNase	No digestion
	9	Yeast tRNA (ssRNA)	No nuclease	No digestion
	10	"	RNase A	Digestion
C	11	"	RNase T1	Digestion
	12	"	RQ1 DNase	No digestion
	13	ØX174/Hae III (dsDNA)	No nuclease	No digestion
	14	"	RNase A	No digestion
D	15	"	RNase T1	No digestion
	16	"	RQ1 DNase	Digestion

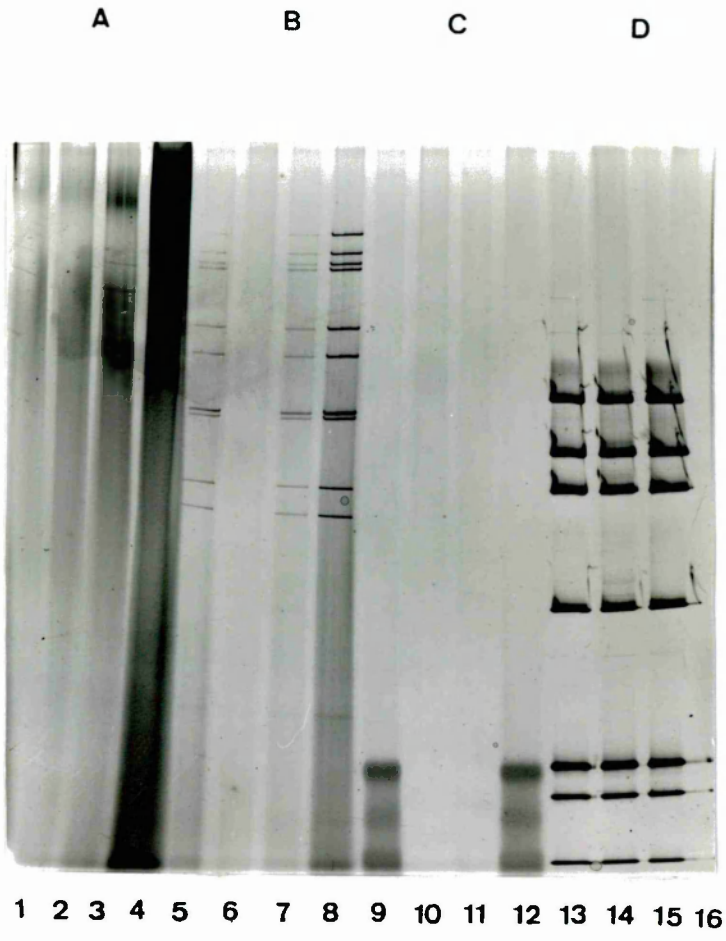


Figure 50. **PAGE analysis of samples from nuclease digestion experiment with RPBV strain R5-9.** Lanes 1 to 16 (see Table 20.).

R11) Determination of the buoyant density in CsCl of rabbit picobirnavirus particles from faeces

The purification of the RPBV from rabbit faeces was performed as described in MM6. Rabbit PBV strain R5-9 was purified as samples were available in reasonably large quantities and the intensity of bands indicated that there was sufficient virus to purify. The PAGE gel shown in Figure 51. shows the sixteen fractions from the CsCl gradient that were extracted as described (see MM6). The picobirnavirus bands were seen in fractions 7, 8 , 9 and 10; with a peak (by staining intensity) in fraction 8, which had a refractive index reading of 1.3695, corresponding to a buoyant density of 1.3800 g/ml. Electron microscopy was also performed on these fractions (results section R12).

Purification was also performed on a RPBV positive faecal sample from rabbit 6 (R6-9). PAGE analysis of the CsCl gradient fractions revealed PBV bands in the fraction with the same buoyant density as R5-9, however the bands were very faint and the gel was not photographed. Purification of other rabbit PBV strains were not performed due to insufficient sample being available.

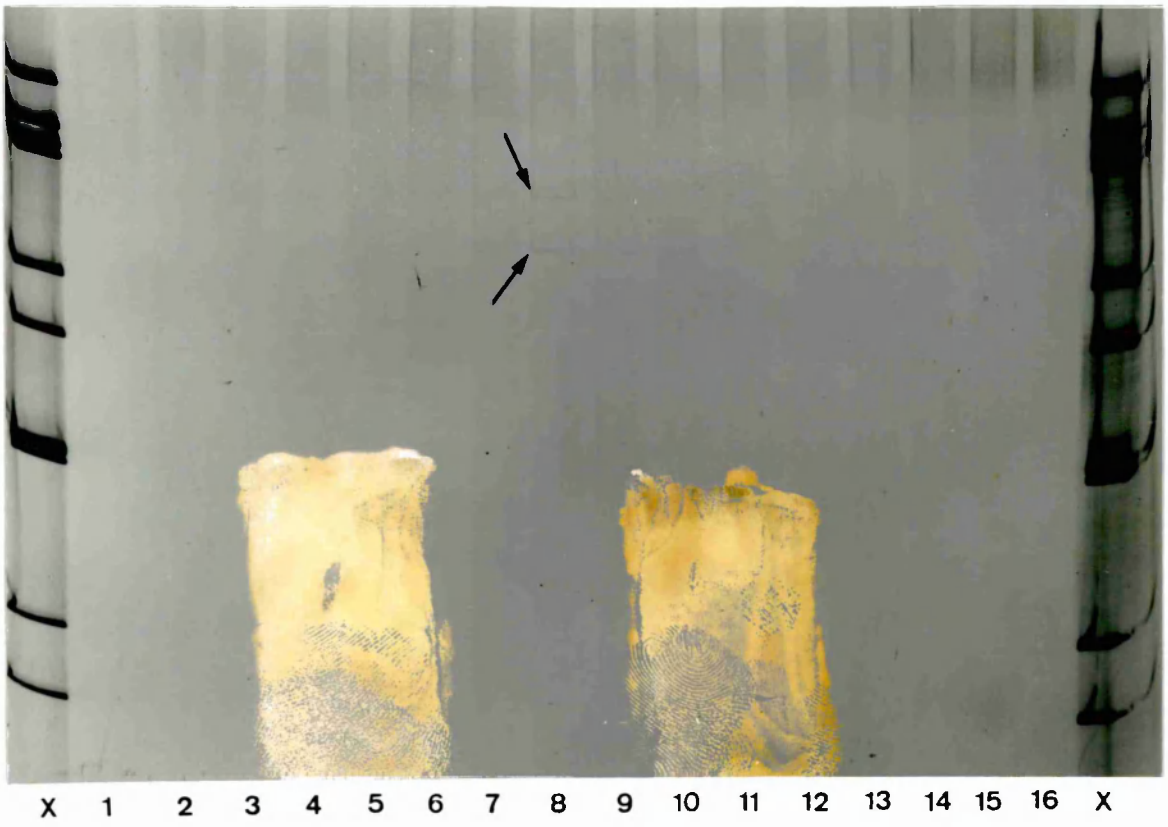


Figure 51. PAGE analysis of nucleic acid from CsCl fractions of rabbit PBV strain R5-9

Lanes 1 to 16 correspond to fraction 1 to 16. lanes 7 to 10 contain RPBV bands (Table 21.). Lane X is SA11 marker.

Table 21. Refractive index and buoyant density determination for CsCl fractions from purification of R5-9

Fraction No.	Refractive Index (RI)	Buoyant Density (g/ml)	PBV Bands (PAGE)
7	1.3685	1.3700	++
8	1.3695	1.3800	+++ (peak)
9	1.3700	1.3850	++
10	1.3710	1.3950	+

R12) Electron microscopy of CsCl gradient fractions from the purification of RPBV strain R5-9

The CsCl gradient fractions from the purification of RPBV strain R5-9 (see MM6) were processed as described in MM7. A PAGE gel was prepared first to determine which fractions were positive for PBV bands (Figure 51.) and fractions 7 to 12 were chosen for examination by electron microscopy. The first virus-like particles seen were small 22nm particles, often called small round particles (SRP) or small round viruses (SRV) or parvovirus-like particles. A large group of these small round particles were seen in fraction 9 (Figure 52.). A second large group of SRPs were seen in fraction 8 (Figure 53.) and alongside them were two larger 32nm particles, which were picobirnavirus-like particles. Further investigation of fraction 8 revealed groups of these 32nm PBV-like particles (Figure 54., 55., 56., and 57.), which appeared to have no distinct morphology, although some of the particles showed a hexagonal structure (Figure 54.). No virus particles were detected in fractions 7, 10, 11 or 12.

The size of the PBV-like particles and the SRPs were measured. The magnification for the negative for Figures 52., to 57. was 62,500 which was corrected to 57,000 (by Dr A Field, VRD).

For the PBV-like particles there were 21 particles which measured 30nm (19%), 84 were 32nm (78%) and 2 were 34nm (1.8%). The total number of particles measured was 107 and the mean diameter was 32nm. The SRPs were also measured and had a mean size of 22nm. The print magnification for the Figures 52. to 57. was 200,000.

Electron microscopy was performed on the pre-inoculation faecal sample from rabbit 5 (R5-0) from study 1, as described for R5-9 and no virus particles were seen (faecal sample was PAGE negative).

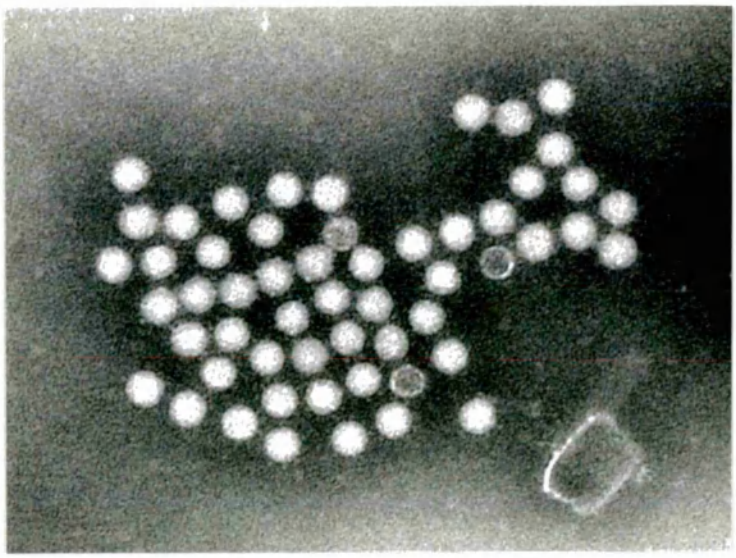


Figure 52. Electron micrograph of small round particles (SRPs) in RPBV strain R5-9 CsCl gradient fraction 9 (Mag. x200,000)

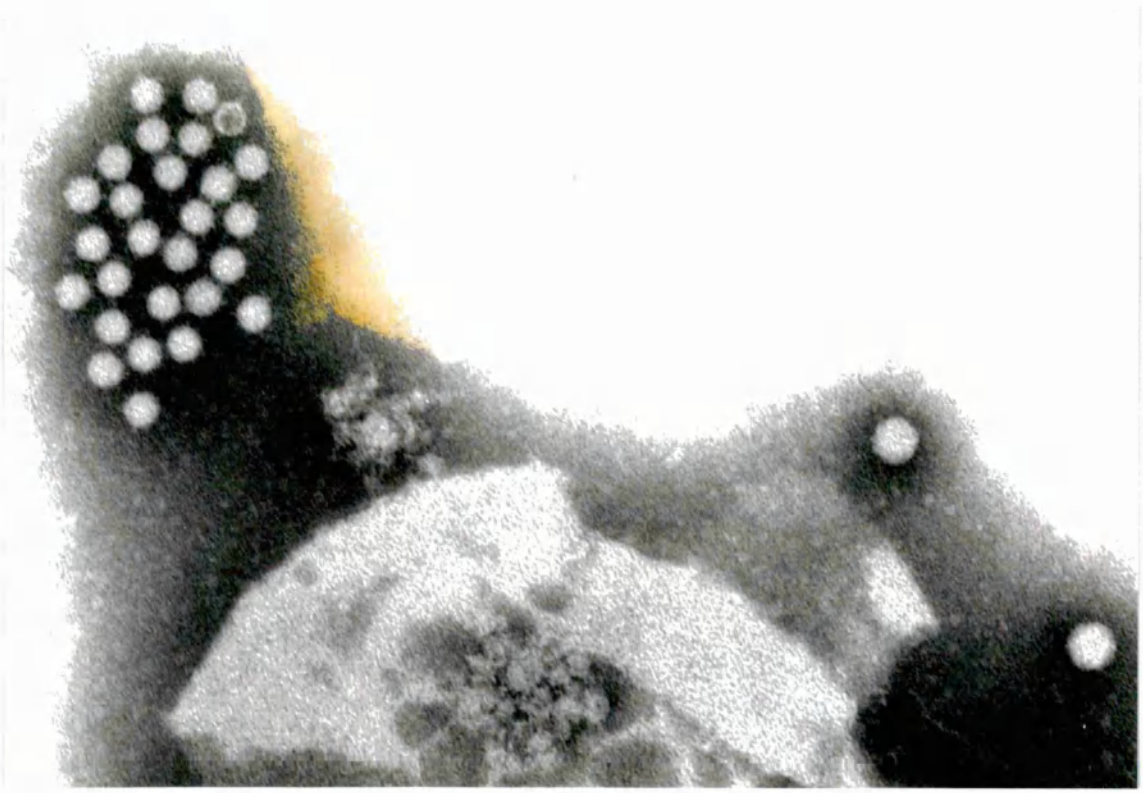


Figure 53. Electron micrograph of a group of SRPs and two larger PBV-like particles in fraction 8 (Mag. x200,000)

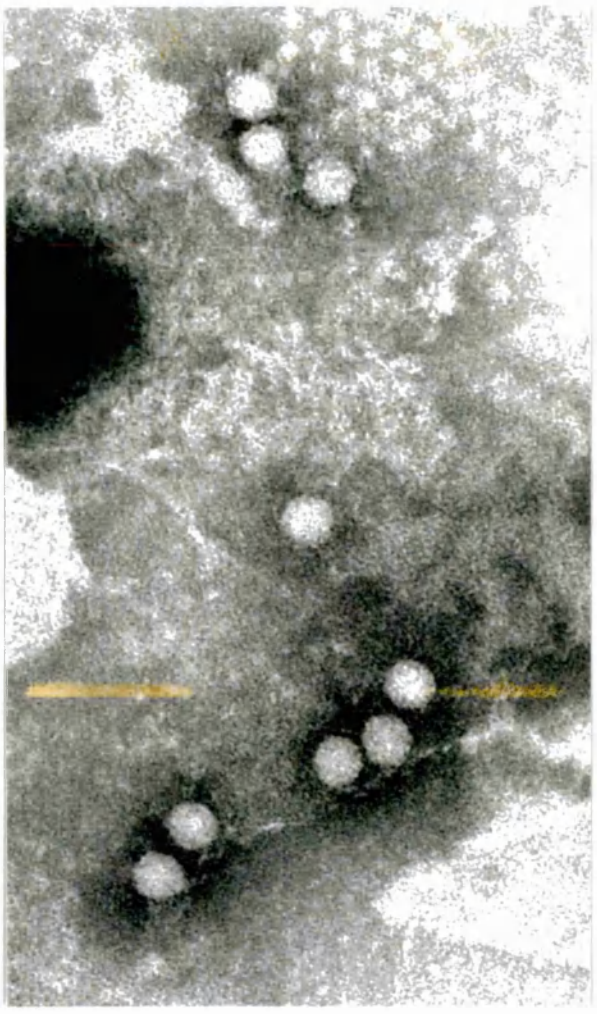


Figure 54. Electron micrograph of PBV-like particles in fraction 8 (Mag. x200,000)

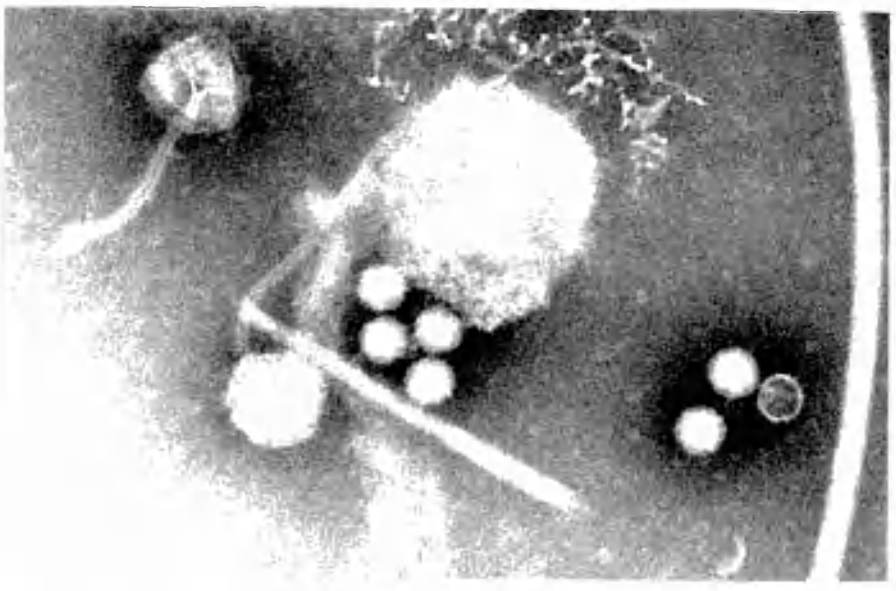


Figure 55. Electron micrograph of a small group of PBV-like particles (Mag. x200,000)

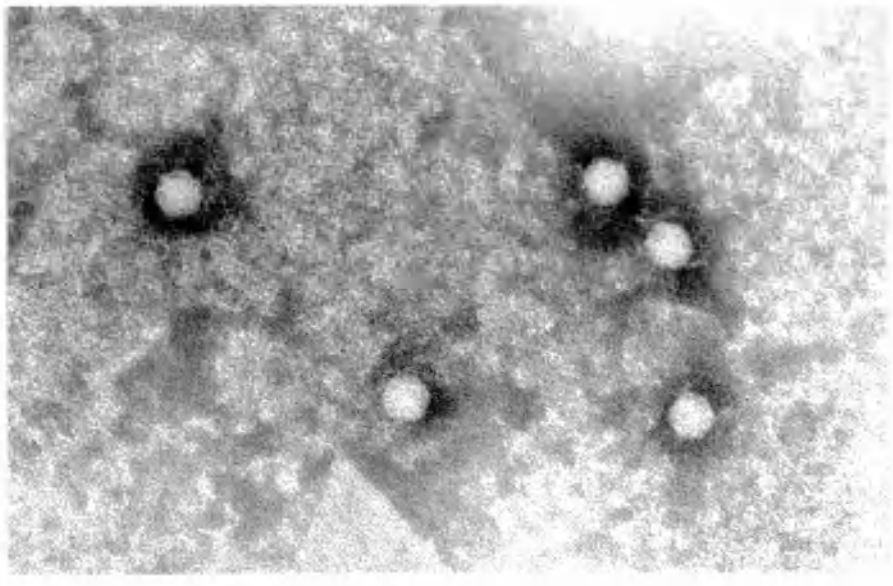


Figure 56. Electron micrograph of several PBV-like particles (Mag. x200,000)

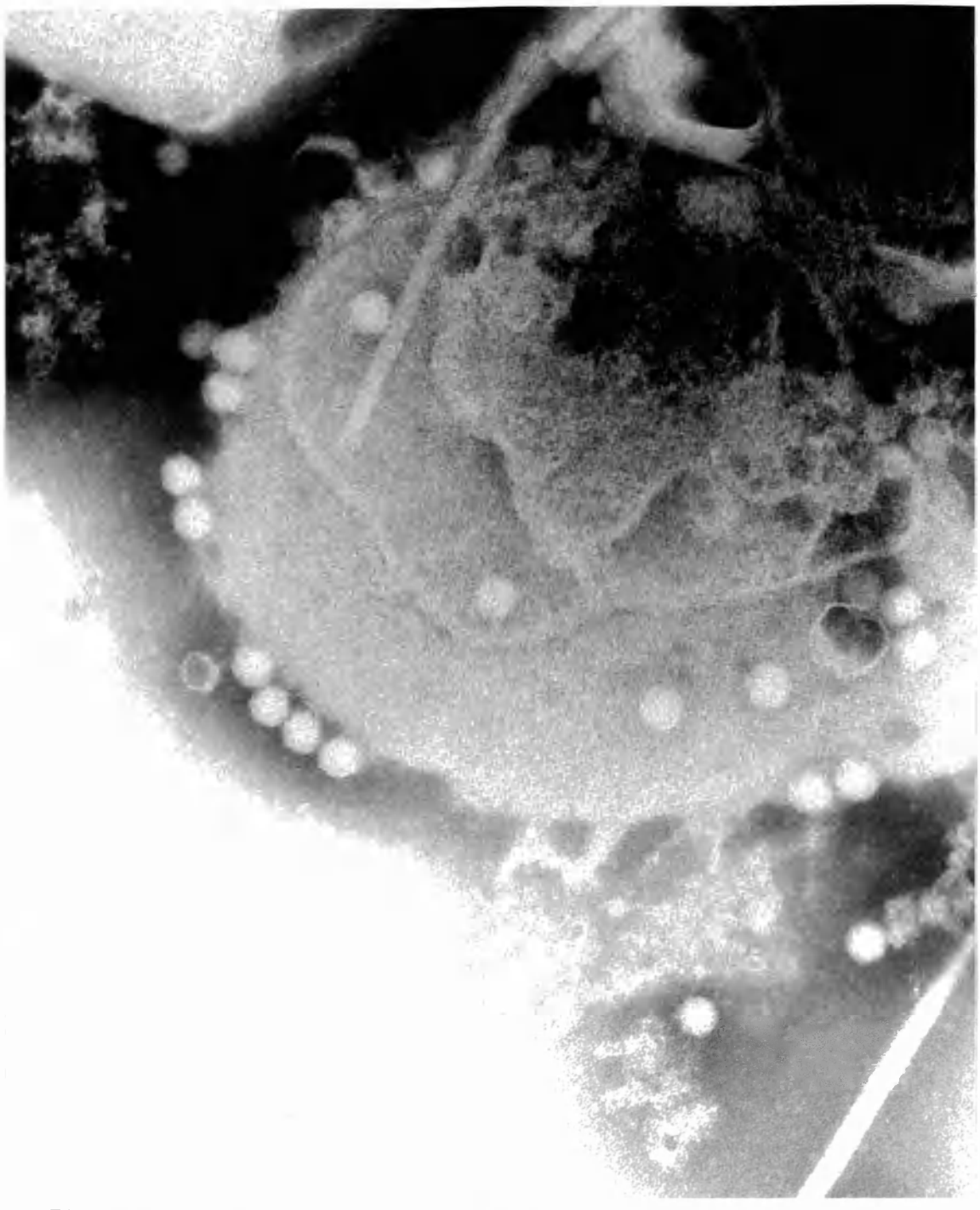


Figure 57. Electron micrograph of a large group of PBV-like particles associated with cellular debris (Mag. x200,000)

R13) Immune electron microscopy of picobirnavirus-like particles from rabbit faeces

This work was undertaken by D.Lewis at Leeds PHL and was previously reported [Gallimore *et al.*, 1993].

Picobirnavirus-like particles were recognised in rabbit 5 day 9 (R5-9) faecal sample by using solid phase immune electron microscopy method (see MM10) with homologous serum. Mainly single particles were observed with little background debris. SPIEM was also used to look for antibody rises in rabbit 5 against homologous virus (R5-9). Grids were coated with a 1:10 dilution of IgG and virus counts (per unit area of grid) of 21 with pre-inoculation serum and 153 with convalescent (day 30) serum were observed; at 1:30 dilution of the IgG fraction the corresponding counts were 5 with the pre-inoculation serum and 290 with the convalescent (day 30) serum.

It was found that recognition of virus particles was greatly facilitated by using the immune clumping method (see MM10) and the number of particles on the grid were further increased. Using this combined SPIEM/immune clumping method the presence of empty or stain-penetrated particles was recognised and these formed clumps with the 'intact' particles (Figure 58.B).

PAGE-positive faecal extracts from study 3 rabbits 6, 7, 10, and 11 were examined by SPIEM using homologous serum. Virus particles were detected in the day 9 faecal sample from rabbit 6. Serum IgG fractions from rabbits 5 (study 1) and rabbits 6, 10, and 11 (study 3) were tested against rabbit 5 (R5-9) virus and rabbit 6 virus (R6-9) using SPIEM/immune clumping, in order to look for the presence of antibody (see Table 22.).

The convalescent serum collected on day 30 from rabbit 5 contained detectable antibody to rabbit 5 virus. The identification of virus clumps with the convalescent (day 30) serum, but not with pre-inoculation serum (Figure 58.A), confirmed the seroconversion demonstrated by SPIEM.

Two different sized particles (not in clumps) were observed in rabbit 6 day 9 (R6-9) faecal sample using day 20 serum from rabbit 6. However, when day 290 serum was used the two different sized particles formed separate clumps (Figure 59. A and B). The larger particle (30nm) was also clumped by both acute and convalescent sera from rabbit 10 and 11 indicating that these rabbits had antibody to this virus prior to the study. The smaller (26nm) particle had a buoyant density of 1.34 g/ml in CsCl, which did not correspond with fractions containing dsRNA bands by PAGE analysis. It may be unrelated to picobirnaviruses.

All three virus particles detected were round or 'slightly hexagonal' with a smooth surface. No subunits or surface structure could be seen. Particles were of similar appearance whether stained with uranyl acetate or uranyl formate, PTA, or whether fixed in 2% glutaraldehyde. Th mean diameters of the particles were: R5-9 particle 30nm, R6-9 large particle 30nm, R6-9 small particle 26nm.

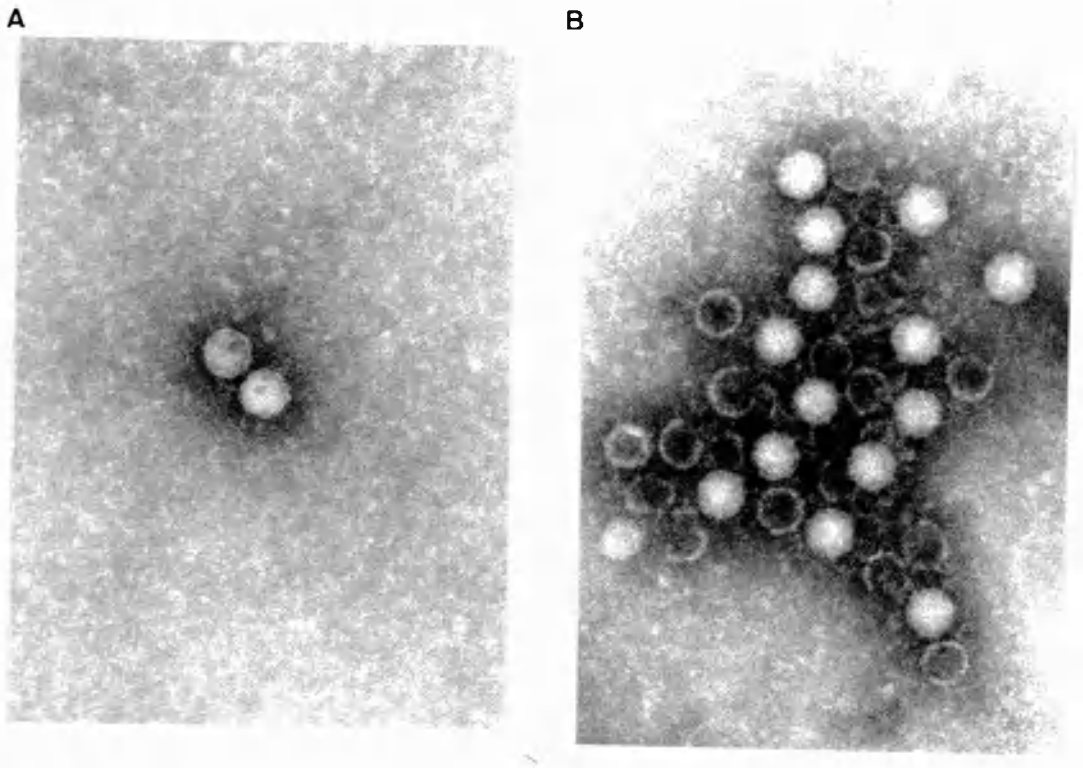


Figure 58. A) Electron micrograph of RPBV strain R5-9 particles using SPIEM/immune clumping of virus and pre-inoculation antibody

B) Electron micrograph of RPBV strain R5-9 particles using SPIEM/immune clumping of virus and convalescent phase antibody

Mag. x200,000 (courtesy of D.Lewis)

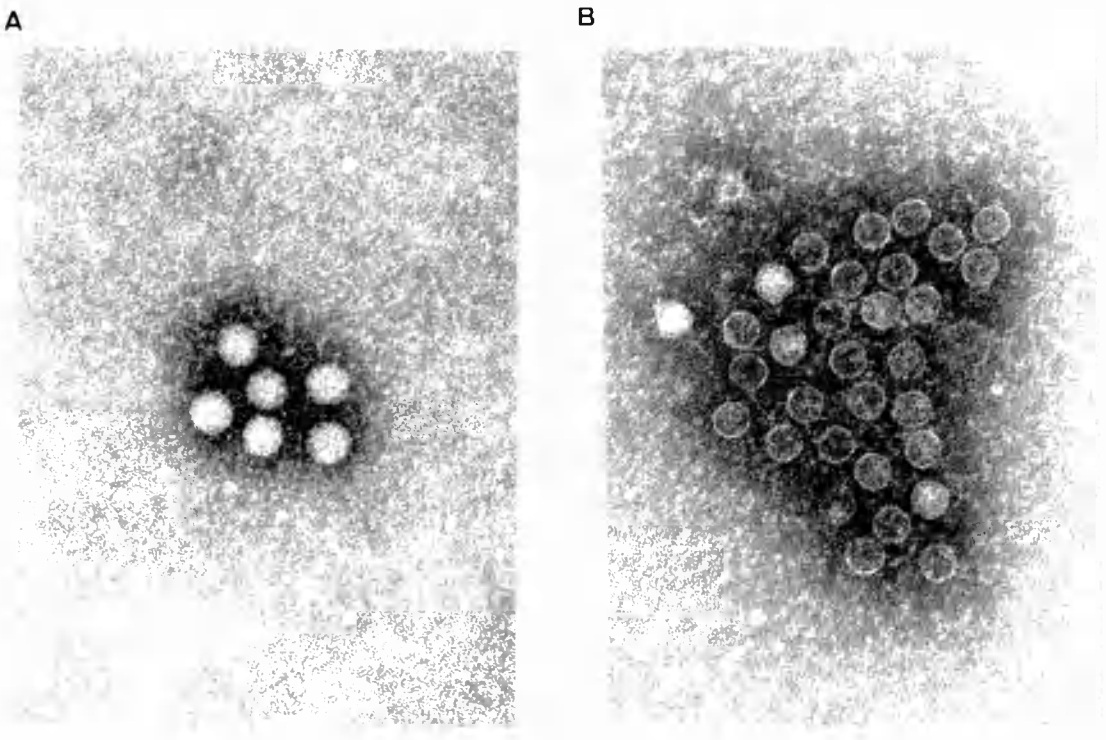


Figure 59. A) Electron micrograph of 30nm PBV-like particles from rabbit 6 (study 3)

B) Electron micrograph of 26nm particles from rabbit 6 (study 3)

The sera used for clumping these virus particles was day 290 sera from rabbit 6.

Mag. x200,000 (courtesy of D.Lewis)

Table 22. Immune clumping EM of two rabbit picobirnavirus preparations with sera from four rabbits

Virus	Rabbit sera							
	RE		R6		R10		R11	
	Pre-innoc	day 30*	d 20	d 290	d 4	d 20	d 4	d 20
35227/89 Rabbit E	30nm particles-no clumps	30nm particles-large clumps	Nd	Nd	Nd	30nm particles-no clumps	Nd	Nd
Rabbit 6 (day-9)	Nd	Nd	30nm particles-no clumps 26nm particles-no clumps	30nm particles-clumps 26nm particles-clumps	30nm particles-clumps 26nm particles-not detected	30nm particles-large clumps 26nm particles-not detected	30nm particles-clumps 26nm particles-not detected	30nm particles-clumps 26nm particles-no clumps

Nd = particles not detected
*day of collection

R14) **Tissue culture studies**

The tissue culture studies involved the inoculation of continuous and primary cell lines with several different picobirnavirus strains.

a) **Human embryo kidney (HEK) cells**

Two strains of picobirnavirus were inoculated into this cell line, one from a human faecal sample (21975/89) and one from a rabbit faecal sample (R5-9). The cells inoculated with 21975/89 showed a slight cytopathic effect (CPE) from day 1 to day 3, but PAGE analysis of the nucleic acid extracted from tissue culture fluid (TCF) from day 1 to day 7 revealed no evidence of picobirnavirus replication. The HEK cells inoculated with PBV strain from the rabbit (R5-9) showed no CPE and PAGE analysis of the TCF at day 7 was negative for the dsRNA bands representative of the picobirnavirus genome.

b) **Human embryonic lung (MRC5)**

The MRC5 cell line was inoculated with PBV strains 21975/89 and R5-9. The cells inoculated with R5-9 strain showed a slight CPE from day 1 to 3, but no virus was seen by PAGE analysis of the TCF. In the cells inoculated with 21975/89, no CPE was observed and no virus was detected in the TCF by PAGE analysis for the detection of the PBV genomic bands.

c) **Primary rabbit kidney cells**

This cell line was inoculated with 4 strains of a picobirnavirus detected in rabbits, R5-9 (study 1), and 3 strains from study 3; R6-9, R7-1 and R10-12 and an uninfected control was used. Two slightly different inoculation procedures were used (see methods)

PAGE analysis of nucleic acid extracted from all of the primary rabbit tissue samples failed to reveal the presence of picobirnavirus nucleic acid.

SECTION 4 Atypical picobirnavirus characterisation

The final section involves the detection and characterisation of novel picobirnaviruses associated with *Cryptosporidium* positive faeces in humans. The term atypical PBV was adopted to differentiate these strains of PBV which have a distinct genome profile that is not characteristic of typical PBV.

R15) a) Demonstration of a unique atypical PBV genome profile
b) Demonstration of the nature of the atypical PBV nucleic acid from human faeces
c) Determination of the buoyant density in CsCl of atypical PBV particles
d) Co-purification studies on *Cryptosporidium* oocysts and atypical picobirnavirus particles

R15) **Detection and characterisation of an atypical picobirnavirus genome profile associated with *Cryptosporidium spp.* positive faeces in humans**

Faecal samples from these patients were analysed by extraction of nucleic acid using the RNAzol/Boom method (see MM2) and PAGE analysis. An atypical PBV genome was detected (see below) and this was followed by preliminary characterisation of the genome and the virus particles.

A) **Detection of an atypical PBV genome profile**

The study was initiated to investigate patients that had faecal samples that were positive for *Cryptosporidium spp.*, as it has been suggested by some workers that these samples may contain picobirnaviruses [Herring, personal communication]. The samples were collected from several PHLS laboratories located in different parts of the country. Demonstration of this unique PBV genome profile can be seen in Figure 60A. & 60B., and 61A & 61B and 62. The term 'atypical' PBV genome profile was used to describe this novel genome profile and to differentiate it from typical PBV genome profiles demonstrated previously. This atypical PBV was only detected in patients faecal samples that were positive for *Cryptosporidium spp.*. In the analysis of 54 *Cryptosporidium* positive faeces by PAGE, 20 (37%) faecal samples were found to be positive for this atypical PBV. This genome profile was not seen in over 1000 faecal samples screened by PAGE for PBV (see R7), except for one patient in the HIV study (strain P5, Figure 33.) and this patient was subsequently found to be positive for *Cryptosporidium spp.*.

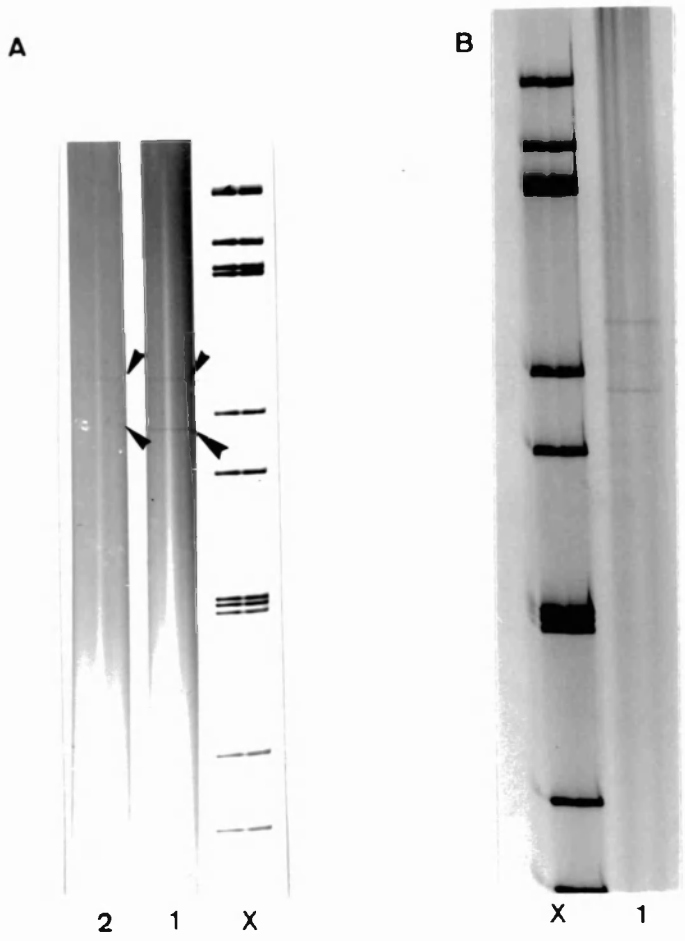


Figure 60A. **Atypical PBV genome profile in two faecal samples**
Lane 1 shows strain G1 and lane 2 strain G2. lane X is SA11.

Figure 60B. **Atypical PBV detected in a HIV-infected patient**
Lane 1 shows strain P5. lane X is SA11.

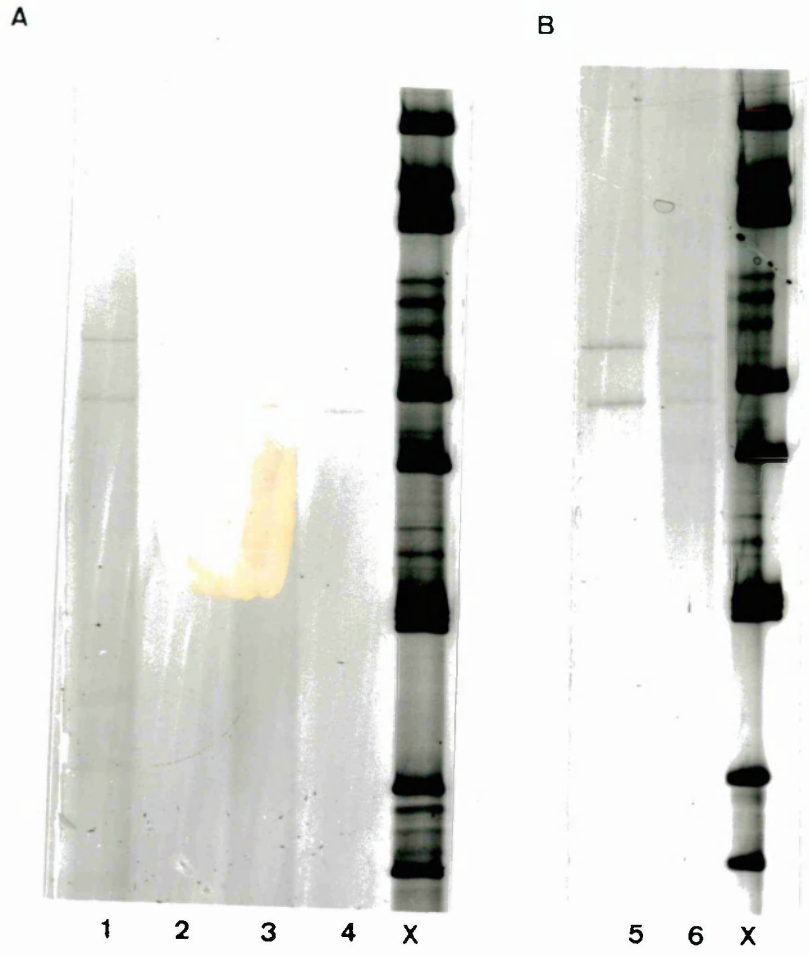


Figure 61A. & 61B. **Atypical PBV genome in six faecal samples**

A) Lanes 1, 2, 3, and 4 show strains RD2, R1, R2, and R4 respectively.

B) Lanes 5 and 6 show strains R5 and R6. Lane X is SA11 (overloaded sample giving strong bands and high background).

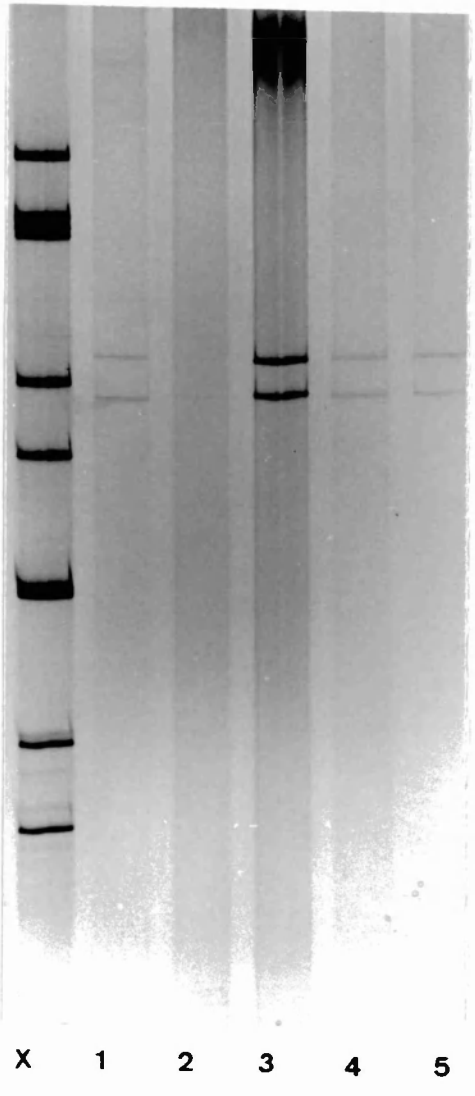


Figure 62. **Demonstration of five further atypical PBV detected in human faeces**

Lanes 1 to 5 show strains R8 to R12, and lane X is SA11.

B) Sizing of atypical picobirnavirus genomic segments

The size of the two genomic segments of three atypical picobirnavirus strains were measured (Table 23.) using the same procedure for the PBV strains (see R2).

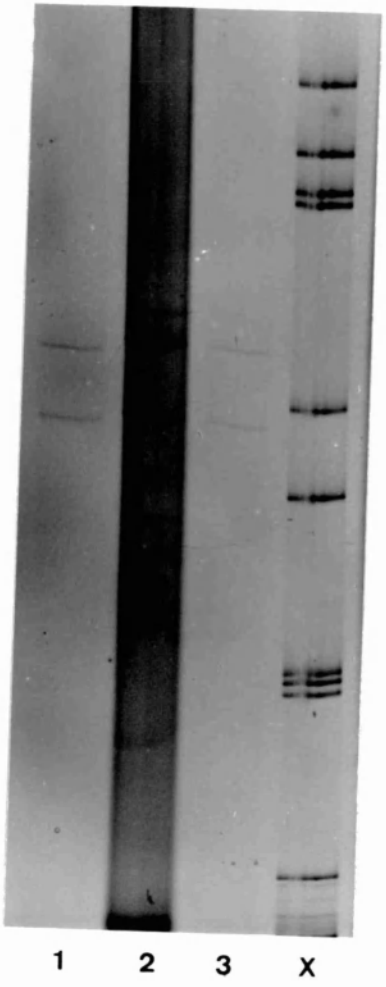


Figure 63. Sizing of atypical picobirnavirus genomic segments using electrophoretic migration in a PAGE gel

Lane 1 shows atypical PBV strain R7, lane 2 strain RD2, lane 3 strain R1 and lane X is SA11 molecular weight marker.

Table 23. **Size estimates of the genomic segments of three atypical PBV**

Atypical PBV	Seg. No.	PBV segment size base pairs
R7	1	1780
	2	1510
RD2	1	1800
	2	1530
R1	1	1780
	2	1500

(data from Figure 63.)

The range for the sizes of the two segments for an atypical PBV are 1.8Kbp to 1.5Kbp for the slow and fast migrating bands as (Table 23.). This range for the genomic segments was seen for all 20 atypical PBV strains detected and all strains had very similar profiles. Five further atypical PBV strains (Figure 62.) were also measured as previously described. Four of these strains had bands which were sized at 1.75Kbp and 1.55Kbp (R8, R10, R11, R12); the other strain R9 measured 1.72Kbp and 1.55Kbp, which had a slightly smaller segment 1. When all eight strains were compared, the average size for the slow migrating band was 1.75Kbp and for the fast migrating band it was 1.55Kbp. The similarity in the genome profile between the atypical PBV strains is demonstrated by the co-electrophoresis of pairs of five of these strains (Figure 64.), and it can be seen that the strains are virtually identical.

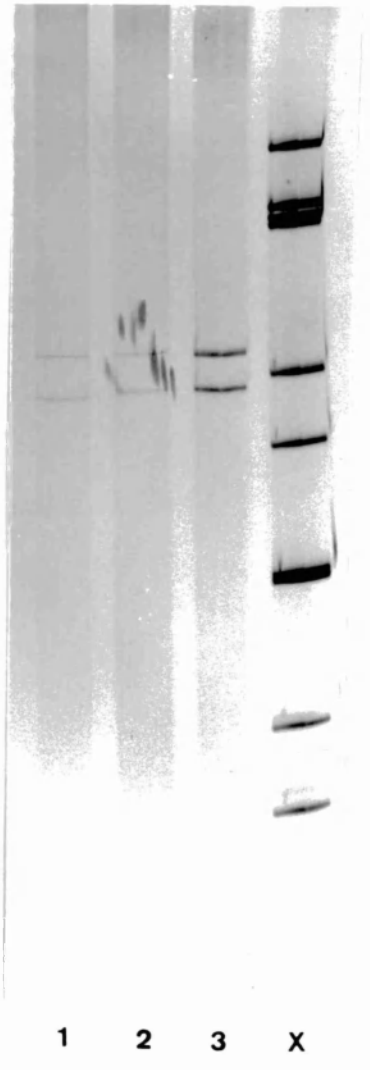


Figure 64. **PAGE demonstrating co-electrophoresis of several atypical PBV strains**

Lane 1 shows R8 + R9, lane 2 R10 + R11, lane 3 R9 + R12. Lane X is SA11

C) Demonstration of the nature of the atypical PBV nucleic acid from human faeces

The characterisation of the atypical PBV nucleic acid represented by the two equimolar bands seen in strain G1 (Figure 60A.). was performed as described in MM5. The results were essentially the same as those described for the PBV strains from human faecal samples described in R3. A PAGE gel demonstrating the effect of different nucleases on atypical PBV strain G1 nucleic acid and control nucleic acid are shown in Figure 65.. and in Table 24. The atypical PBV strain G1 nucleic acid had the same digestion profile as rotavirus SA11 (dsRNA). and the two segments were confirmed to be dsRNA. Another atypical PBV strain (R6) was also shown to have two segments consisting of dsRNA.

Table 24. **Atypical PBV strain G1 nucleic acid digestion data**

	Lane	Nucleic acid	Nuclease	Result
	1	SA11 (dsRNA)	No nuclease	No digestion
	2	"	RNase A	Digestion
A	3	"	RNase T1	No digestion
	4	"	RQ1 DNase	No digestion
	5	APBV strain G1	No nuclease	No digestion
	6	"	RNase A	Digestion
B	7	"	RNase T1	No digestion
	8	"	RQ1 DNase	No digestion
	9	Yeast tRNA (ssRNA)	No nuclease	No digestion
	10	"	RNase A	Digestion
C	11	"	RQ1 DNase	No digestion
	12	"	RNase T1	Digestion
	13	1 Kb ladder (dsDNA)	No nuclease	No digestion
	14	"	RNase A	No digestion
D	15	"	RQ1 DNase	Digestion
	16	"	RNase T1	No digestion

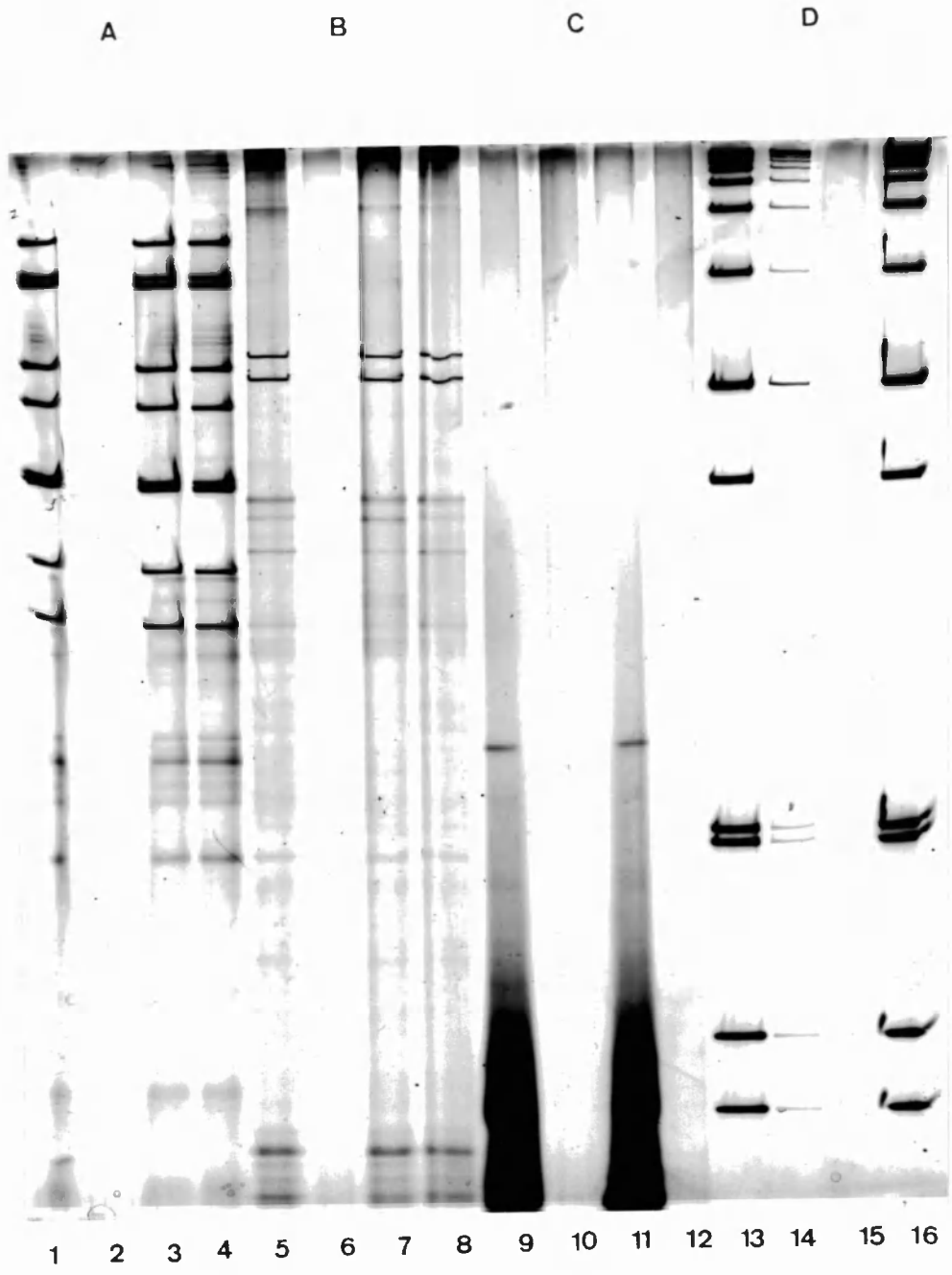


Figure 65. PAGE analysis of samples from nuclease digestion experiment with atypical PBV strain G1. Lanes 1 to 16 (see Table 24.).

D) **Determination of the buoyant density in CsCl of atypical PBV particles from human faeces**

The purification of an atypical PBV from human faeces was performed as described in MM6. APBV strain G1 was selected because it was strongly positive by PAGE (Figure 65.) and the faecal sample was available in sufficient quantities. The PAGE gel shown in Figure 66. shows the sixteen fractions from the CsCl gradient that were extracted as described (see MM6). The atypical PBV bands were seen in fractions 6, 7, and 8; with a peak (by staining intensity) in fraction 7. The fraction 7 sample from the CsCl gradient which contained atypical PBV bands, had a refractive index reading of 1.3695, corresponding to a buoyant density of 1.3800 g/ml. The fractions were also examined by electron microscopy (as described in R12), but no virus particles were seen in any of the fractions.

No other atypical PBV strains were purified due to insufficient material.

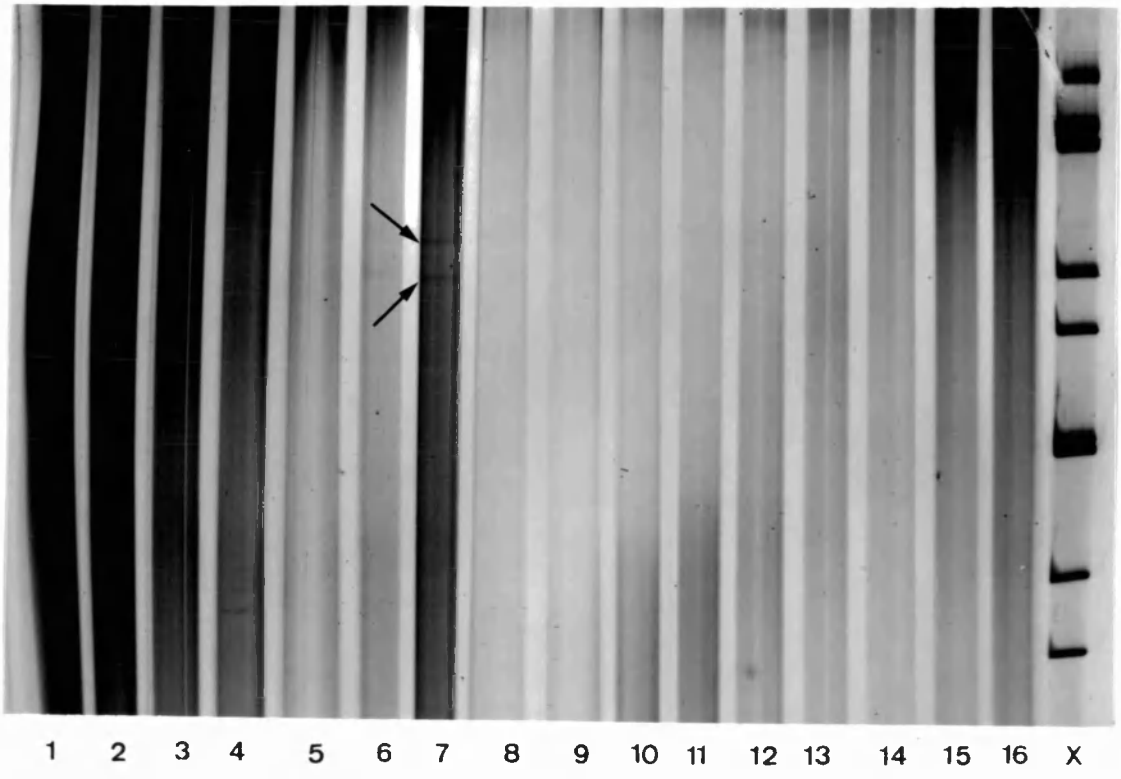


Figure 66. PAGE analysis of nucleic acid from CsCl fractions of atypical PBV strain G1

Lanes 1 to 16 correspond to fraction 1 to 16, with lanes 6 to 8 containing APBV bands (see Table 25.). Lane X is SA11 marker.

Table 25. Refractive index and buoyant density determination for CsCl fractions from purification of atypical PBV strain G1

Fraction No.	Refractive Index (RI)	Buoyant Density (g/ml)	Atypical PBV Bands (PAGE)
6	1.3690	1.3750	+
7	1.3695	1.3800	++ (peak)
8	1.3705	1.3900	+

E) Co-purification of *Cryptosporidium* oocysts and picobirnavirus particles

PAGE analysis was performed on faecal material from three atypical picobirnavirus specimens (strains R9, R11 and R12), following a method to concentrate the *Cryptosporidium* oocysts in the specimens. PAGE analysis was performed on 500ul 10% faecal extracts of the three samples prior to concentration and on 200ul of the 250ul final pellet obtained after oocyst concentration. The PAGE gel clearly shows the presence of the atypical PBV bands in the 10% faecal extract before oocyst concentration, but there is no evidence of atypical PBV bands in the concentrated oocyst preparations (Figure 67.). The three faecal samples were shown to contain oocysts in the final pellet by immunofluorescence staining [Casemore, 1991], (data not shown). It would therefore appear that the atypical picobirnaviruses do not co-purify with *Cryptosporidium* oocysts, or the virus was lost during the extraction procedure.

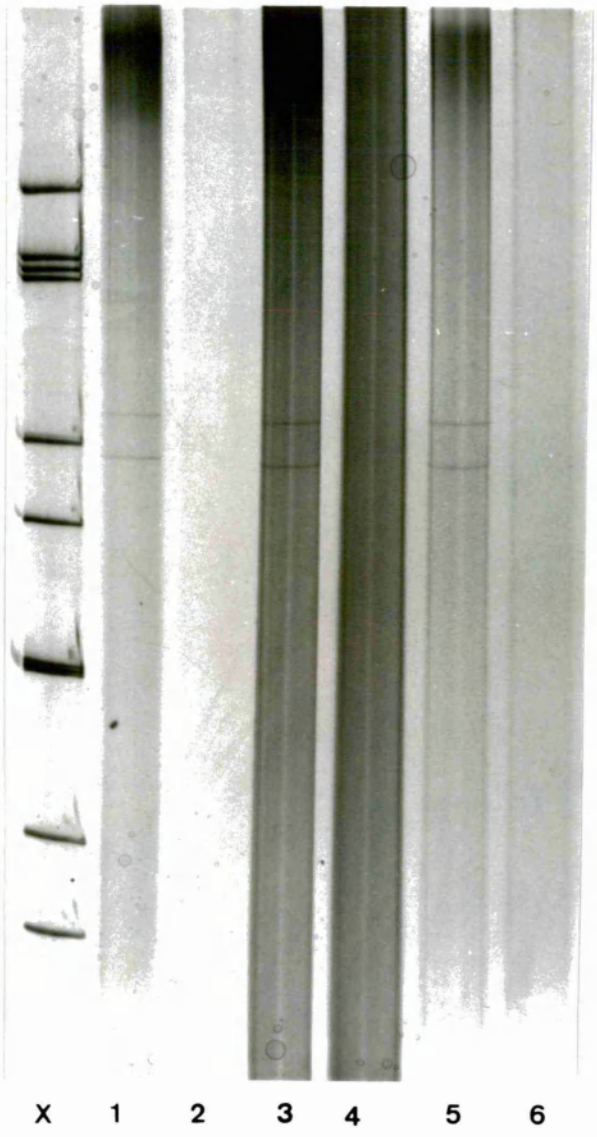


Figure 67. **PAGE analysis of atypical picobirnaviruses in faecal samples from a *Cryptosporidium* oocyst concentration procedure.**

Lane 1, 3, and 5 are 10% faecal extracts of atypical PBV strains R9, R11, and R12. Lanes 2, 4, and 6 are R9, R11, and R12 pellets from the oocyst concentration procedure. Lane X shows SA11 marker.

DISCUSSION

The discussion of this thesis will deal with the following areas: 1) The characterisation of picobirnaviruses; 2) The epidemiology of a) human picobirnaviruses, and b) rabbit picobirnaviruses; and 3) Summary and future prospects with picobirnaviruses and viral gastroenteritis.

D1) Characterisation of picobirnaviruses

The first part of this study demonstrated that the two equimolar bands that were detected PAGE and silver staining were the genome of the newly described group of bisegmented dsRNA viruses termed picobirnaviruses [Pereira *et al.*, 1988a & 1988b]. The criteria for picobirnavirus characterisation were as follows: two equimolar segments of dsRNA in the size range 2.5 Kbp to 1.5 Kbp for the slow and fast migrating bands respectively; buoyant density in CsCl in the range 1.38 to 1.40 g/ml; and particles approximately 35nm in size with no distinct surface morphology.

The initial detection of picobirnaviruses was by PAGE analysis of of nucleic acid extracted from human faecal samples, which was subsequently used as the standard method of detection for these viruses. In the early PAGE analyses, a group A rotavirus (later replaced by rotavirus SA11) was used for size estimation of possible PBV genomic bands.

The human PBV strain 21975/89 (Figure 5.) was used as a PBV type strain to characterise the genome, as it had a typical PBV genome profile by PAGE analysis. The genomic segments were sized by comparison with SA11 [Mitchell and Both, 1990] and found to be 2.5 Kbp and 1.73 Kbp [Gallimore *et al.*, 1993]. This fulfilled the

criteria set by Pereira et al., [1988a & 1988b]. The demonstration that the two bands were double-stranded RNA, was by nuclease digestion (Figure 9.).

The next stage was to show that the bisegmented dsRNA genome was a viral genome. This was attempted by using CsCl gradient centrifugation of PBV positive faecal samples to purify virus particles. PAGE analysis of CsCl gradient fractions revealed PBV bands in fractions which corresponded to a buoyant density in the range 1.38 to 1.4 g/ml (Figure 10. & 11.). Electron microscopy was performed on these fractions, but no particles were seen. However the demonstration of the two dsRNA bands characteristic of PBV in a CsCl gradient at a buoyant density described for PBV (Pereira *et al.*, 1988a & 1988b), strongly suggested that they were the genome of this virus.

Direct electron microscopy was used to detect PBV particles in PAGE positive faecal samples to determine whether these particles had the characteristic size and morphology. Several faecal samples were examined and two representative electron micrographs are shown in Figure 13.& 15.. The particles seen were in the size range 35 to 40nm, similar to that described by Pereira *et al.*, [1988a & 1988b], and they also lacked a distinctive morphology. The electron microscopy of PBV in human faeces will be discussed in more detail later.

Now that the characteristics of a PBV were established, a more detailed analysis of the PBV genomic profiles was undertaken.

The segments of sixteen PBV strains were measured and had an average size of 2.46 Kbp for segment 1 (slow migrating band) and 1.74 Kbp for segment 2 (fast migrating band). The ten PBV strains illustrated in Figure 6. displayed a degree of diversity in genome profiles. The average total genome size of these strains was 4.2 Kbp, which was very similar to that of seven strains detected in HIV-infected patients (4.16 Kbp) shown in Table 7. It would appear therefore that even if there is some variation in genome profiles, picobirnavirus strains detected in human faeces may be related as their total genome sizes are very similar; however sequencing of the genome would be needed to investigate how closely the strains are related.

The genomic sizes of several different groups of bisegmented dsRNA viruses are compared in Table 26. and are given as molecular weight (Mol. Wt) in daltons x 10^{-6} and as kilo base pairs (Kbp). This was calculated assuming an average molecular weight of 660 daltons per base pair [Both *et al.*, 1982], or measured by electrophoretic migration in PAGE, and the genomic profiles of dsRNA viruses in general are illustrated diagramatically in Figure 68.

During attempts to pass PBV in rabbits, several rabbit PBV strains were detected which were presumed to be endogenous viruses already present in the rabbit host (discussed later). Three types of genomic profile of PBV were detected in rabbits: narrow, 2.14 Kbp and 1.93 Kbp, intermediate; 2.30 Kbp and 1.90 Kbp; and wide, 2.63 Kbp and 1.84 Kbp (Figure 48.) and the rabbit strains that were classed in each of the profiles were all very similar [Gallimore *et al.*, 1993]. The average total genome size of rabbit PBV (4.5 Kbp) is similar to that of human PBV (4.2 Kbp).

Table 26. Relationships in the size of the genomic segments of different bisegmented dsRNA viruses

Virus	Mol. Wt ($x10^6$)	Total M Wt	Kbp	Total Kbp
Birnaviruses	2.30, 2.50	4.80	3.38, 3.78	7.26
Picobirnaviruses (human faeces)	1.15, 1.62	2.77	1.74, 2.46	4.20
Picobirnaviruses (rabbit faeces)				
I(N)	1.27, 1.40	2.67	1.93, 2.14	4.07
II(I)	1.25, 1.52	2.77	1.90, 2.30	4.20
III(W)	1.20, 1.74	2.94	1.84, 2.63	4.47
Atypical Picobirnaviruses (human faeces)	1.00, 1.16	2.16	1.55, 1.75	3.30
Cryptoviruses (plant viruses)				
WCCV I	1.00, 1.20	2.20	1.50, 1.82	3.32
WCCV II	1.40, 1.50	2.90	2.10, 2.30	4.40
Partiviridae (mycoviruses)	0.90, 1.60	2.50	1.36, 2.42	3.78

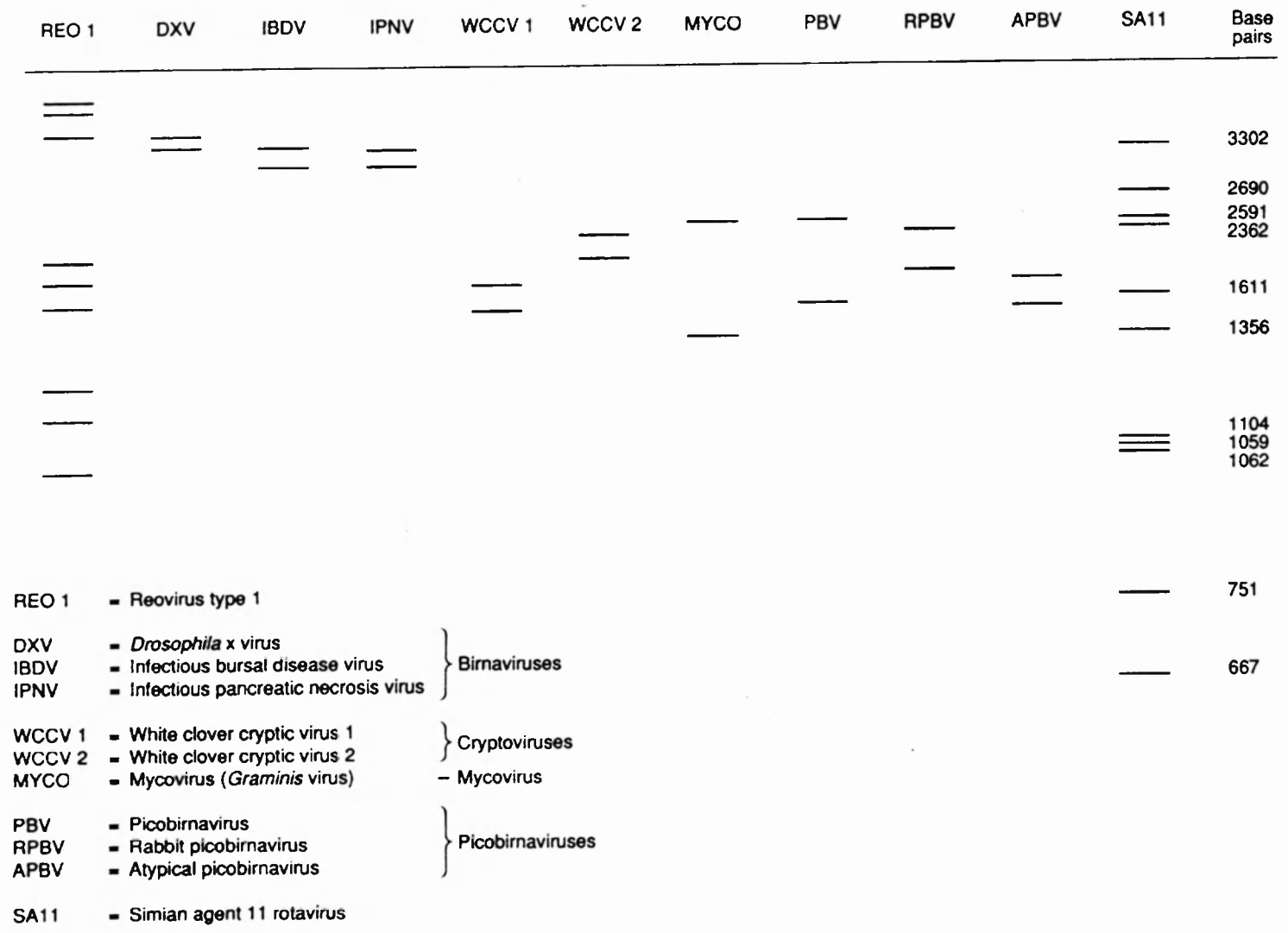


Figure 68. Diagramatic representation of the genome profiles of several segmented dsRNA viruses

The genome profile of a typical picobirnavirus is illustrated in Figure 68. and is contrasted to other types of picobirnaviruses, birnaviruses, mycoviruses, cryptoviruses, reoviruses and rotaviruses, to show how PBV genome profiles compare to other segmented dsRNA viruses.

The two birnaviruses, IPNV [Dobos *et al.*, 1983] and IBDV [Becht, 1980], have quite distinct genome profiles (Figure 68.) and their two genomic segments in total are at least 3 Kbp larger than the other bisegmented dsRNA viruses (Table 26.). In addition, birna-type viruses detected in cattle [Vanopdenbosch and Wellemans, 1989; Vanopdenbosch and Wellemans, 1990] have a genome profile similar to birnaviruses; but the genome sizes have not been clearly documented.

The only other group of bisegmented dsRNA viruses that have a similar genome size to picobirnaviruses are the cryptic plant viruses or cryptoviruses [Boccardo et al., 1987]. The white clover cryptic virus 2 (WCCV2) has a total genome size similar to that of human PBV. The two individual segments of WCCV2 are however, 2.3 Kbp and 2.1 Kbp (Table 26.) and this type of profile was not seen with picobirnaviruses detected in human faeces, which have two segments of approximately 2.5 Kbp and 1.5 Kbp.

Studies on picobirnaviruses in various animal species [Pereira *et al.*, 1988b; Grohman *et al.*, 1993; and Ludert *et al.*, 1991] have demonstrated genomic variation. Gatti *et al.* [1989] compared genomic profiles of 16 strains of porcine PBV. Although there was some variation in the segment 1 (2.56 to 2.18 Kbp) and the segment 2 (1.61 to 1.31 Kbp) of all sixteen strains, the overall genomic profile of PBV in porcine faeces was similar and showed a 'wide' profile. Ludert *et al.* [1991] also demonstrated a 'wide' PBV genomic profile in five porcine PBV strains. The segment 1 bands were in the range 2.4 to 2.6

Kbp and the segment 2 bands were 1.7 to 1.9 Kbp. This wide profile was also the predominant pattern among PBV strains detected in the HIV-infected patients study (Figure 33.), and in other patient groups (Figure 6.). PBV detected in chickens by Monteiro *et al.* [1991], also appears to have a genome profile similar to that described in pigs and humans. This uniformity of genome profile was not, however, seen with PBV detected in rabbit faeces, where three distinct genome profiles (narrow, intermediate, and wide) were detected [Gallimore *et al.*, 1993] (Figure 48.).

Having characterised the genome, attempts were made to identify PBV virus particles. There were two different types of PBV-like particles seen by direct electron microscopy of human faeces. The first PBV-like particles which were 38 to 40 nm in size and had a pronounced rim (Figures 12., 13., and 15.) and the second type of PBV-like particles which were 35 to 38 nm with no surface morphology (Figures 14., 16., and 18.). The PBV-like particles from sample 10494/92 (Figures 12. and 13.) appear to have a similar morphology to those described in rats by Pereira *et al.* [1988b, Fig. 2.c.], which are slightly smaller, but also have a pronounced rim. The rim seen in PBV-like particles in sample 10494/92 (Figures 12. and 13.) and sample 9122/92 (Figure 15.) were present in intact, partially disintegrating and empty particles. The PBV-like particles seen in sample 3380/92 (Figure 14.) appear to be similar to those seen in porcine faeces by Ludert *et al.* [1991, Fig.5.]. PBV-like particles were seen in some human faeces that were shown to be negative by PAGE for PBV bands, these samples were 9122/91 (Figure 15.), 10876/92 (Figure 16.), and 3378/92 (Figure 17.). These observations suggest that the genomic bands demonstrated by PAGE and the particles observed may be unrelated. However, it may be that insufficient

quantities of PBV were present in these samples to be detected by PAGE, but by chance a large group of particles were seen by EM in sample 10876/92 (PAGE negative). A similar large group of particles were seen in sample 3380/92 which was PAGE positive.

It is highly likely that PBV-like particles have been observed by other workers without being recognised. For example, in the report on the viral flora of faeces as seen by electron microscopy by Flewett *et al.* [1974], 40nm isometric particles with a hexagonal outline were seen [Flewett *et al.*,1974, Fig.7.]. These virus-like particles would appear to be morphologically similar to the PBV-like particles seen in sample 10494/92 (Figures 12. and 13.).

By contrast, in the studies of rabbit picobirnaviruses it was possible to link demonstration of the dsRNA PBV genome with virus particles. RPBV strain R5-9 genomic bands were demonstrated in CsCl gradient fractions, with the peak fraction (by staining intensity) corresponding to a buoyant density of 1.38 g/ml. Electron microscopy was performed on the peak fraction from R5-9 CsCl gradient and PBV-like particles were seen in large numbers. The virus particles (Figure 53. to 57.) were approximately 32nm in diameter and have a smooth surface with some particles showing a hexagonal structure (Figure 54.) and were similar to those described by Pereira *et al.* [1988a]. Smaller 22nm small round particles or parvovirus-like particles sometimes in large groups (Figures 52.) were seen in the same EM field as two larger PBV-like particles (Figure 53.). This clearly shows the difference in size between the two types of virus particle. It was not surprising to see parvovirus-like particles in the same CsCl fraction as PBV-like particles, as full parvovirus particles have a buoyant density in CsCl of between 1.39 and 1.42 g/ml. Parvoviruses have been described in a number of different

animals including rabbits [Matsunaga *et al.*, 1977]. It is not known whether the observed parvovirus was a rabbit parvovirus or a human parvovirus acquired with the inoculum (human faecal sample 21975/89). Parvovirus-like particles were seen in sample 21975/89 (data not shown), but they were unlikely to be that seen in rabbit faeces because parvoviruses are generally host specific [Ridpath and Mengeling, 1988].

Rabbit PBV-like particles (Figure 57.), appear to be morphologically similar to those seen in human faeces (e.g. sample 3380/92, Figure 14.). This is to be expected since members of other gastroenteritis virus groups (rotaviruses, small round structured viruses or small round viruses) and other viruses are morphologically similar [Theil, 1990; Greenberg *et al.*, 1990; Bridger, 1990], regardless of the host they infect.

A novel group of bisegmented dsRNA viruses were detected in *Cryptosporidium spp.* positive faeces from humans and were termed atypical picobirnaviruses (APBV). The APBV detected all had very similar genome profiles, consisting of two dsRNA segments which were sized at 1.75 Kbp and 1.55 Kbp (total 3.3 Kbp). This genome profile was not characteristic of typical PBV or birnaviruses, though the profile was closer to PBV, hence the name atypical PBV. The unique sizes of the two segments seen in APBV (Figure 68.) and the uniformity of their genome profiles may lead to them being recognised as a novel group of bisegmented dsRNA viruses or a subgroup of picobirnaviruses.

As with typical PBV, atypical picobirnaviruses have a total genome size which was similar to one of the cryptic plant viruses, white clover cryptic virus 1 (WCCV1). APBV have two segments of 1.75 Kbp and 1.55 Kbp which are similar to the two segments of WCCV1 (1.82 Kbp

and 1.50 Kbp). The WCCV1 total genome size is 3.32 Kbp (Table 26.) and the APBV total genome is 3.30 Kbp. By contrast, typical PBV have two segments which individually are different in size to those of another cryptic virus, WCCV2, but similar in total. The exact relationship of APBV to WCCV1 may only be determined when sequence information becomes available.

The use of genome analysis by PAGE for the detection of picobirnaviruses is still in its infancy and further studies are needed to fully understand the significance of these bisegmented dsRNA genome profiles. With other well studied dsRNA viruses such as rotaviruses [Estes and Cohen, 1989], and birnaviruses [Dobos and Roberts, 1983; Kibenge *et al.*, 1988] genome analysis has been important in virus detection and molecular epidemiology [Estes *et al.*, 1984]. The term 'molecular epidemiology' implies the use of biochemical methods to characterise viruses in an effort to understand how they spread and cause infections.

The epidemiology of picobirnaviruses is discussed next.

D2) **Epidemiology of picobirnaviruses**

a) Human picobirnaviruses

Epidemiological studies on human picobirnaviruses described in this thesis were based on the detection in faeces by polyacrylamide gel electrophoresis of the two picobirnavirus genomic segments in the range 2.5 Kbp and 1.5 Kbp. The initial study was a retrospective analysis of faecal samples collected mainly from adults and elderly patients in outbreaks of gastroenteritis between 1982-89 (retrospective study). In these samples a number of viruses were detected by electron microscopy (Figure 23.), with small round structured viruses the predominant group. This is consistent with the age of the patients as SRSV mainly affect adults and the elderly.

Nearly all the PBV strains detected in this retrospective study were detected in elderly patients (Figure 22.), with no PBV found in younger adults and only 1 PBV in a child. This is quite different from PBV detection in the outbreak samples collected between 1991-93 (prospective study). Here, 53% of PBV strains were detected in adult samples from food associated outbreaks in hotels and restaurants and general hospital wards, whereas 40% of PBV were detected in elderly patients (geriatric homes etc.) and 3.5% in children from schools (Figure 27.). The prevalence of PBV by patient age in all the epidemiological studies is given in Table 27.

It was found that for elderly patients in hospital geriatric wards the prevalence of PBV was 7% (retrospective study) and 10% (prospective study). With the elderly who live in old peoples homes there was a three fold decrease in the detection of PBV in the 1991-93 samples compared to the 1982-89 samples. With the adult samples from hotels and restaurants there was an increase in PBV detection from 0% (1982-89 samples) to 16% (1991-93 samples).

Table 27. **Comparison of PBV prevalence by age in the epidemiological studies**

Patient group	Age	No. of samples	No. of PBV by PAGE	Prevalence of PBV (%)
1982-89	Elderley	200	11	5.5%
samples	Adults	109	0	0
	Children	41	1	2.4%
1991-93	Elderley	166	12	7%
samples	Adults	134	16	12%
	Children	28	1	4%
Hospitalised	Elderley	9	1	11%
patients	Adults	19	1	5%
	Children	45	8	18%
HIV-infected patients	Adults	133	7	5%
Sporadics	Unknown	69	3	4.3%

In outbreaks in schools (children only) rates of 2% to 4% were noted and may represent the endemic prevalence rate in this age group in this setting. In outbreaks from geriatric wards the overall prevalence was 7.5%, compared to 4.5% in the elderly in old peoples homes. The higher prevalence in geriatric wards in hospitals may reflect the more crowded conditions and more transient populations.

A PBV prevalence of 16% was found in adults involved in outbreaks of gastroenteritis in hotels and restaurants in samples studied prospectively from 1991-93. It is not known why there were no PBV detected in the samples from 1982-89, as similar numbers of samples from this group of patients were tested. It is difficult to interpret the 3.5% rate seen in sporadic cases (Figure 26.), but this may represent the prevalence of PBV infection in this setting.

With a PBV prevalence of 16% in outbreak samples, and 3.5% in sporadic cases, this may indicate that PBV is more prevalent in outbreaks. With outbreaks, the mode of transmission may be food-borne (possibly with SRSV, discussed later), as the adults involved were ill generally 24 to 48 hours after attending a catered function. With the elderly patients in geriatric wards, food may also be the source of infection. However, when one patient has diarrhoea and/or vomiting on a ward the other patients in close proximity to the index case may develop symptoms by cross-infection.

The overall incidence in the 1982-89 samples was 3.5%, whereas in the 1991-93 samples it was 9%. It is possible that the increase in detection of PBV between these two groups of samples reflects either, sample storage or nucleic acid extraction. The 1982-89 samples were stored at -40^{o}C, whereas the 1991-93 samples were stored at 4^{o}C. It is not known what effect storage temperature of the sample has on the detection of PBV by PAGE analysis, though it is unlikely that this is

the reason for the difference in prevalence of PBV detection. A more probable explanation is the method of nucleic acid extraction used on the two groups of samples. With the 1982-89 samples, the nucleic acid was extracted using the phenol/chloroform method, whereas the 1991-93 samples were extracted using the 'Boom' method. It has been shown with hospitalised patients' samples [Gallimore *et al.*, 1995a] and HIV-infected patients samples (both of which are discussed later), that the 'Boom' method of extraction increases the yield of nucleic acid and also produces a 'cleaner' preparation. The combination of these two factors enhances the visualisation of faint PBV genomic bands by PAGE analysis and silver staining. These low levels of PBV nucleic acid are probably at sub-nanogram quantities and are at the limits of detection by silver staining [Berry and Samuel, 1982].

In the hospitalised patients study (Table 13.), patients of different ages and with a variety of clinical diagnoses were examined with respect to PBV prevalence rates. The clinical diagnoses or main presenting illnesses were grouped into several categories to determine whether the detection of PBV was related to gastroenteric (GE) symptoms or non-gastroenteric symptoms. It appeared that PBV circulated quite widely (13% to 14%) in patients both with and without gastroenteritis. This along with the HIV-infected patients study (to be discussed) suggests that there is no evidence that PBV causes gastroenteritis in these patients. There appeared to be no correlation between a particular illness and detection of PBV in non-gastroenteritis patients. The incidence of PBV in elderly patients (11%) was similar to that for geriatric ward patients described earlier. The prevalence in infants (13%) and children (20%) may reflect a higher level of PBV infection in these age groups in hospitals, but a larger number of samples from these age groups would

need to be analysed to determine whether this was a significant difference. The adult group had a PBV incidence of 5% which is similar to that seen in adults from general hospital wards found in the gastroenteritis outbreak samples (1991-93).

When the hospitalised patients samples were originally tested using the phenol/chloroform method only 3/79 (4%) of the samples were positive for PBV. However, when the same samples were retested using the 'Boom' method, 11/79 (14%) PBV were detected which was a three-fold increase in detection. This again strongly suggests that the 'Boom' method is a more sensitive method than phenol/chloroform for the extraction of PBV dsRNA from faecal samples.

The other major group of patients that were examined were HIV-infected patients with and without diarrhoea. In this study there were 133 patients from whom 237 samples were collected. The patient samples were divided into three groups; diarrhoea, no diarrhoea and not known. It was found that patients without diarrhoea had a greater prevalence of PBV in their faeces than those with diarrhoea (Figure 35.). It is difficult to interpret this result as the number of patients without diarrhoea was small compared to those with diarrhoea. However, the overall prevalence of PBV in HIV-infected patients (5%) appears similar to that previously described for adults in general hospital wards and the findings do not indicate that HIV-infected patients are more likely to have PBV in their faeces. By contrast, the study by Grohman *et al.* [1993] demonstrated an prevalence rate of 9% in HIV-infected patients with diarrhoea and 2% in those without diarrhoea. However, samples in the two studies were not extracted in the same way, so it is difficult to make a comparison. When faecal samples in our HIV-infected patients study were originally extracted by the phenol/chloroform method, about 1%

of samples contained PBV. The same samples were then retested using the 'Boom' method and there was a greater than three-fold increase in the detection of PBV. In the HIV-infected patients study of Grohman *et al.* [1993], 4 PBV (1.8%) were detected in 222 patients using a 10% faecal extract and 16 PBV (7.2%), using an ultra-centrifuge pellet which represented a 15 to 30-fold concentration of virus from the stool compared to using a 10% faecal extract. They showed a four-fold increase in the prevalence of PBV by concentrating the 10% faecal extracts. A similar increase was observed in this study, when the 'Boom' method of nucleic acid extraction was adopted, without an ultracentrifuge concentration step, which is a more elaborate and time consuming procedure. This demonstrates how specimen preparation technique can affect the outcome of a study.

The epidemiology of PBV in sporadic cases of gastroenteritis was investigated using faecal samples from patients with diarrhoea. The samples were referred for bacteriological testing by their General Practioner and were then examined by PAGE for the presence of PBV genome. The prevalence of PBV in these samples using the phenol/chloroform method of nucleic acid extraction was 4.3%.

In the study by Grohman *et al.* [1993], one patient was shown to be excreting the same PBV strain over several months. A similar finding was made in our laboratory. The PBV strain detected was identical in all samples collected from an individual over the four month period (Figure 36.). This long term excretion suggests that the PBV strain was replicating in the gut, rather than passively acquired from the diet. However, it is not known whether this replication was in the host tissue or whether the PBV was replicating in other micro-organisms, such as bacteria, fungi or protozoa present in the gut. Although plant viruses have been demonstrated to be resistant to

degradation in the human alimentary tract [Tomlinson *et al.*, 1982]. it is unlikely that PBV are plant viruses as the quantities of virus being ingested would have to be implausibly great to be detected in the faeces by PAGE. With cryptic plant viruses the yield of virus has been estimated at between 100 and 200ug virus/Kg of leaf [Boccardo *et al.*, 1987]. Therefore with a scenario where 1g of faeces had the same quantity of virus as 1g of leaf, the faecal sample would contain approximately 100ng of virus. If 500ul of a 10% faecal extract was used for PAGE it would thus contain 5ng of virus. In this situation, the host species would have to ingest large quantities (several kilogrammes) of infected white clover for the virus to be detected in faecal samples. Therefore it is unlikely that PBV are cryptic plant viruses, but, the possibility that they may be a plant virus that is present in large quantities in a common food cannot be totally discounted. The only food material that was tested for PBV was the feed pellets given to rabbits, and these were shown to be PAGE negative (data not shown).

The examination of faeces from patients with a diagnosis of *Cryptosporidium* infection revealed the presence of atypical picobirnaviruses [Gallimore *et al.* 1995b]. In this study, 54 such samples from various PHLS laboratories were tested and 20 (37%) contained a bisegmented dsRNA genome profile. The genome was smaller than that of a typical PBV and all 20 strains had virtually the same profile. These PBV were tentively named atypical PBV (APBV). This high prevalence rate may indicate an association of these atypical PBV with *Cryptosporidium spp.* positive faeces. It is therefore possible that these APBV are protozoan viruses.

The genome of APBV is not characteristic of any of the dsRNA viruses of protozoa which have been described in the following

organisms; *Trichomonas vaginalis* [Wang and Wang, 1986a; Khoshnan and Alderete, 1993], *Giardia lamblia* [Wang and Wang, 1986b] and *Babesia bovis* [Johnston *et al.*, 1991]. All of these viruses of protozoa when originally classified shared several common features, [Wang and Wang, 1991]: a) all are dsRNA viruses with a non-segmented genome of 5Kb to 7Kb; and b) all are spherical or icosahedral in shape with a diameter of 30 to 40 nm. However, recent studies have shown that the *Trichomonas vaginalis* virus is multisegmented, having three segments in the range 4.8 Kbp to 4.3 Kbp [Khoshnan and Alderete, 1993]. APBV are distinct from these viruses and, as no viruses of *Cryptosporidia* have yet been described, the atypical PBV described here may be the first virus of this protozoan group.

Preliminary evidence suggests that APBV do not infect or are not retained in the oocyst stage of *Cryptosporidium* development, because no PBV nucleic acid could be detected in concentrated oocyst material (Figure 67.). It is possible therefore that the APBV infect one of the other developmental stages such as the sporozoite or merozoite. They may also be a co-incidental finding and have no association with *Cryptosporidia*. Further studies are needed to confirm that atypical PBV are viruses of *Cryptosporidium spp.*.

The mode of transmission of picobirnaviruses to the human host is not known though it is possibly by the faecal-oral route. This has been confirmed for group A rotaviruses [Kapikian *et al.* 1983]. Rotaviruses have been shown to be relatively stable in aerosols in a rotating drum [Sattar *et al.*, 1984], indicating that airborne transmission may also be possible.

The pathogenesis of viral infection of the gastrointestinal tract may take several different forms [Blacklow and Cukor, 1982]. One type of disease is due to direct local damage produced by viral

multiplication in the gastrointestinal tract, and this has been described for rotaviruses and Norwalk-like viruses [Christensen, 1989]. There is no evidence that PBV cause disease in this way as they have been demonstrated in patients with and without diarrhoea. A second mode of infection is demonstrated by viruses that appear to replicate in the gut without producing symptoms but, following viraemia, spread to other target organs, where they produce significant disease. These viruses include members of the enterovirus group, eg. poliovirus and hepatitis A virus [Dienstag *et al.*, 1978]. It is possible that picobirnaviruses are similar to enteroviruses in that they may replicate in the gut without causing symptoms, but cause a yet unrecognised illness at another site in the body. A third mode of gastrointestinal tract infection is found with viruses that would not normally infect the gut. Cytomegalovirus (CMV), for example, is one of the most common and potentially serious opportunistic pathogens of the gastrointestinal tract in AIDS. Cytomegalovirus-associated colitis is characterised by diarrhoea, fever and abdominal pain [Smith *et al.*, 1992]. CMV is rarely (if ever) seen in faeces [Madeley, 1989], and is diagnosed by biopsy of the colon. It would seem unlikely, therefore that this is the mode of PBV infection in the human host, as PBV is not infrequently seen in faeces.

b) Rabbit picobirnaviruses

Tissue culture was investigated in attempts to develop an *in vitro* culture system for propagating PBV, but these studies were unsuccessful. It was then decided to investigate an *in vivo* propagation system for PBV in small animals. The only animals in which PBV was detected were rabbits. The first rabbit that showed evidence of PBV in faecal samples was rabbit 5 (R5-9) in study 1 (Figure 39.). There was no evidence that the human PBV (strain 21975/89) from the inoculum had replicated in rabbit 5 and it would thus appear that the PBV detected (R5-9) was a co-incidental finding of an endogenous virus.

The strain R5-9 was then used as the inoculum in study 3, which involved the oral inoculation of newly weaned rabbits. In this study a number of rabbits excreted PBV with different genomic profiles (Table 19.). In rabbit 7 a genome profile similar to the inoculum was found in the faeces on day 1 and 2, which was followed by excretion of a PBV with a different genome profile on day 5 to 11. It is possible that this represents replication of the R5-9 RPBV in rabbit 7 as the genomic profiles of R5-9 and R7-1 strains are only slightly different [Gallimore *et al.*, 1993, see Figure 2.]. This would need to be confirmed by sequencing. It is more likely that the PBV strains found in the study 3 rabbits were present before inoculation and again were endogenous viruses. In only one rabbit was a PBV detected in day 0 faeces (Figure 40.), and in the other rabbits, PBV excretion occurred several days after the inoculation. In study 4 the RPBV strain R5-9 was re-inoculated into two rabbits from study 2 and 8 rabbits from study 3 (Table 17.). This resulted in the detection of two different PBV strains in rabbit 17 (Figure 45.) and rabbit 10 (Figure 46.). It is not known why some rabbits excreted PBV for only

a few days, for example rabbit 5 (R-9) in study 1 (Figure 39.), whereas others excreted virus for up to 10 days (rabbit 7 and rabbit 10) from study 3 (Figures 42. and 43.). In rabbit 6 from study 3, excretion lasted for up to 44 days which was unique for any of the PBV infected rabbits, though long term excretion had been demonstrated in human faeces (e.g. PBV strain 21975/89).

It might be expected that the rabbits that had PBV strains with similar genome profiles had been kept together and that they had acquired the PBV by cross-infection. This does not appear to be the case for the study 3 rabbits. They were caged individually and two pairs of rabbits which had similar genome profiles, rabbits 6 and 7, and 10 and 11 were in different rooms. The possibility that the virus was carried from one room to another by a member of staff, cannot be discounted. Another potential source of PBV was the animal feed, which comes in the form of dried pellets, but PAGE analysis of a 10% extract of the feed pellets did not reveal any PBV (data not shown).

With hindsight, baseline faecal samples should have been collected for several days or weeks, prior to the rabbits being inoculated to monitor for any concurrent PBV excretion. Although rabbits may excrete PBV, it would appear that the majority of them began to excrete PBV at least four days after inoculation. This would suggest that the inocula (Table 19.) which were a faecal extract from rabbit 5 (RPBV strain R5-9) or a faecal extract from a human (PBV strain 21975/89) may have stimulated excretion of PBV in the rabbit.

The view that excretion of PBV in rabbits was triggered by oral inoculation of a faecal extract (which was positive for PBV) may be supported by the concept of 'enteritis complex', which was proposed in the study on rotavirus infection in rabbits by Hambraeus *et al.*

[1989]. The term describes that diarrhoea in the rabbit is mediated by an interplay of different microbial agents such as; coccidia, bacteria, and virus [Whitney, 1976; Peeters *et al.*, 1982; Peeters *et al.*, 1984] in the gut. The idea is that oral inoculation with a faecal sample (PBV positive or possibly not) may disturb the microbial flora in the gut of a rabbit, this results in the replication and excretion of the PBV that is already present. To confirm this, control rabbits would have to be inoculated with faecal extracts that were PBV negative, while other rabbits are inoculated with PBV positive faecal samples.

Where the replication takes place is important in determining whether PBV is a mammalian virus or a virus of a micro-organism e.g bacteria, fungi or protozoa, however this has yet to be determined. Using immune electron microscopy, it was shown in rabbit 5 (R5-9) that there was an immune response temporally associated with virus excretion (Figure 58.A and B). This is consistent with the view that these are vertebrate viruses. Immune electron microscopy also demonstrated that acute and convalescent sera from rabbits 10 and 11 (Table 22.) contained antibody to the 30nm virus particles isolated from rabbit 6 (Figure 59.A.). In rabbit 6, antibody to the homologous isolate was detected in a blood sample collected several months after completion of the study, but not in the day 20 convalescent sample, suggesting that the antibody response developed more than 14 days after virus excretion was first identified. The SPIEM method has proved useful in demonstrating serotype differences with small round structured viruses [Lewis *et al.*, 1988]. The observations from the IEM data was consistent with at least two antigenically different picobirnaviruses circulating in rabbits 5 (R5-9) and 6 (R6-9), which are also clearly distinguishable by genome profile (Figure 39. and

41.). The demonstration of an immune response and virus excretion, however, are not definitive proof that PBV are vertebrate viruses. Antibody to the bacteriophage ØX174 has been demonstrated in humans in newborn infants [Uhr *et al.*, 1962], in normal adults [Peacock *et al.*, 1973], and in immunodeficient patients [Ochs *et al.*, 1971; Hamblin *et al.*, 1975]. In the study by Hamblin *et al.* [1975] one patient had antibody present in their serum prior to inoculation with the bacteriophage, which showed that humans acquire antibody to bacteriophages naturally.

Only one bacteriophage with a dsRNA genome, has been reported Ø6 [Mindich, 1988]. The Ø6 dsRNA genome, however, consists of three segments which are 6.37 Kbp, 4.06 Kbp and 2.95 Kbp in size [Pereira, 1991]. The virus particles are also unlike PBV, as they are about 75nm in diameter, with a flexible envelope and a dodecahedral capsid of 60nm [Ackermann, 1991].

The pathogenesis of PBV in rabbits is unclear. The majority of the rabbit faecal samples that were positive for PBV were of a formed nature but slightly moist. However, faecal samples from day 8, 9, and 10 from rabbit 5 (study 1) were not formed and were moist. It is difficult to interpret clinical signs with regard to faeces in rabbits as previously demonstrated by work using a rabbit as model for rotavirus infection [Conner *et al.*, 1988]. It was shown that rabbits normally excrete a soft moist faeces (night faeces), which may be indistinguishable from a soft stool caused by rotavirus infection. Rabbits also have an effective water absorptive capacity in the large intestine and previous studies in rabbits with enterotoxigenic *Escherichia coli* showed that diarrhoea was not observed unless the caecum was removed [Evans *et al.*, 1982].

Transmission and pathogenicity of PBV in rabbits may have similarities to those of rotavirus in rabbits. In a study on the pathogenicity of rotavirus by Thouless *et al.* [1988], it was shown that a stool filtrate from a dead rabbit in the outbreak was no more pathogenic than tissue culture adapted virus. This suggests that factors other than virulence of the particular rotavirus isolate were involved in the severity of infection. Co-pathogens or immunological resistance of rabbits, resulting either from hereditary factors or from animal husbandry practices such as diet, hygiene, or stress, may be involved. This may explain why some rabbits seemed to excrete PBV for a short period and others for a longer time. Another interesting observation by Thouless *et al.* [1988] was with regard to transmission of virus. They observed infection by rotavirus in sham-infected or uninoculated rabbits. Uninoculated does and offspring housed with experimentally infected litters became infected as expected, but uninoculated rabbits which were housed in a separate rack room also became infected. It was concluded that they were unlikely to have been infected by fomites by the usual faecal-oral route and may have been infected by airborne virus. Airborne transmission has been described for epizootic diarrhoea (rotavirus) of infant mice [Kraft, 1957], in which uninoculated mice housed in separate cages in the same room developed diarrhoea about 2 days after the appearance of diarrhoea in experimentally infected mice. The agent was unknown at the time but was later described as a rotavirus [Petric *et al.*, 1978]. The virus was thought not to be spread by human contact as gloves and new forceps were used when handling the mice. It is possible that airborne transmission of PBV in rabbits occurred and this would explain why rabbits in different rooms excreted PBV with similar genome profiles in their faeces.

D3) Summary and future prospects with picobirnaviruses and viral gastroenteritis

It is still uncertain whether picobirnaviruses are viruses which infect man and other animals and whether they are responsible for causing disease in the host. The review on viruses and diarrhoea by Madeley [1983], describes how difficult it is to fulfil Koch's postulates with viruses and disease [Rivers, 1936], and in particular to prove causation with those viruses that are associated with gastroenteritis. It has been shown by several workers that immunodeficient patients can excrete more than one enteric virus in their faeces [Chrystie *et al.*, 1982; Wood *et al.*, 1988]. In the epidemiology studies described in this thesis small round structured viruses were seen in patients that also had picobirnaviruses. Dual detection was demonstrated in sixteen different outbreak samples between 1982 and 1993 (Table 12. and 13.). It is likely, therefore, that in this situation the primary cause of gastroenteritis was the SRSV which is a known human pathogen. Excretion of known gastroenteritis viruses without causing overt disease has been shown with rotaviruses in man [Chrystie *et al.*, 1975; Murphy *et al.*, 1977; Madeley *et al.*, 1978; Chrystie *et al.*, 1978]. This was, however, documented in the newborn, whereas disease normally associated with rotaviruses occurs in older children (6 months to 5 years) [Madeley, 1979]. With picobirnaviruses a correlation between detection of virus and disease could not be demonstrated. We do not know whether picobirnaviruses cause gastroenteritis in a particular age group and it would be interesting to look at a larger number of faecal samples, including samples from newborn babies and young children.

A question still to be resolved is whether typical picobirnaviruses and atypical picobirnaviruses are mammalian viruses or possibly protozoan viruses, mycoviruses or bacteriophages. There is some evidence to suggest that PBV are mammalian viruses. Firstly, there was demonstration of long term excretion of PBV in a human and a rabbit. This would indicate that replication is taking place and it is therefore unlikely that the virus is a plant virus ingested with food. However, it is not known whether this replication occurs in the host tissue or in a gut micro-organism. Secondly, in the rabbit studies there was demonstration of a seroconversion in one rabbit coinciding with excretion of a PBV in the same rabbit. Again this is not definitive evidence that PBV is a mammalian virus, since antibody to bacteriophage has been detected in humans [Hamblin *et al.*, 1975].

Attempts were made to see whether PBV was a bacteriophage by inoculating broth cultures with PBV positive faeces, extracting nucleic acid from the broth and performing PAGE analysis (data not shown). No PBV RNA was detected. Alternatively, the strong association of APBV with *Cryptosporidia spp.* may indicate that APBV and perhaps even typical PBV are protozoan viruses.

The molecular cloning of a human, rabbit or an atypical PBV may lead to the identity of the PBV host being unveiled.

Some preliminary studies using molecular biology techniques [Green *et al.*, 1992], where rabbit PBV genomic segments were purified from faecal material, have allowed us to perform complementary DNA (cDNA) cloning of the genomic RNA from rabbit (R5-9) strain of picobirnavirus in order to further characterise a PBV genome and develop molecular probes for use in epidemiological studies in humans.

The clones were shown by northern blot hybridisation to be derived from segment 1 or the slow migrating band (2.3 Kbp), and no clones were detected for segment 2. The segment 1 clones were sequenced and oligonucleotide primers were designed and a 1.6 Kbp region of segment 1 was amplified by PCR. The cDNA's of the 5'and 3' terminal regions were obtained by the RACE (rapid amplification of cDNA ends) procedure. The rabbit PBV segment 1 was shown to be 2.311 Kbp in length. Northern blot hybridisations with cDNA probes covering the whole of segment 1 were performed on the dsRNA of a number of rabbit and human PBV strains; no cross hybridisation could be demonstrated, showing a lack of sequence homology between viruses.

If this work was to be continued, it would be interesting to clone the atypical picobirnaviruses as they appear to be genomically similar by PAGE analysis and therefore one would expect them to be related at the sequence level. Cloning of a typical human picobirnavirus would also be required in order to develop more sensitive detection assays such as nucleic acid hybridisation and PCR and may also lead to expression of recombinant PBV proteins and the development of an EIA-based serology test. This would allow investigations of the epidemiology of these viruses and their possible role in pathogenesis in the human host.

The work in this thesis has demonstrated the importance of methodology when attempting to identify novel viruses. It was shown that the use of an elaborate nucleic extraction method [Boom *et al.*, 1990] significantly increased the detection of PBV by silver staining and PAGE. Therefore the combination of an improved extraction technique, and a sensitive silver staining technique (both of which can be seen to have improved from the beginning of this work to the APBV studies at the end) would be the probable explanation why other

workers appear not to have recognised this group of viruses. Although, there was no evidence of an association between PBV and disease in humans, a significant amount of new information has been obtained on picobirnaviruses in humans and rabbits, and on the atypical picobirnaviruses associated with *Cryptosporidium* positive faeces from humans.

APPENDIX

Appendix

A1) Buffers and stains

Tris/Ca

20mM Tris-Hcl

10mM $NaCl_2$

3mM $CaCl_2$

pH 8.0

TE

10mM Tris-Hcl

1mM EDTA (pH 8.0)

pH 7.4

L6

120g guanidinium thiocynate (GTC)

100ml 0.1M Tris-HCl pH 6.4

22ml 0.2M EDTA pH 8.0

2.6g Triton X-100

L2

120g GTC

100ml 0.1M Tris-HCL pH 6.4

Polyacrylamide gel electrophoresis buffers and reagents

Resolving gel buffer

Trizma (Sigma) 36.3g dissolved in distilled water and pH adjusted to 8.9, and diluted to 100ml with distilled water.

Stacking gel buffer

Trizma (Sigma) 5.98g dissolved in distilled water and pH adjusted to 6.7, and diluted to 100ml with distilled water.

Electrophoresis running buffer

Trizma (Sigma) 6g

Glycine (Merck-BDH) 28.8g

Dissolved and diluted to 1L with distilled water.

Sample buffer

Stacking gel buffer 10ml

Glycerol (United States Biochemical-USB) 4ml

Bromophenol blue (Merck-BDH) 20mg

Dissolved and diluted to 20ml with distilled water.

Silver stain

0.37g Silver nitrate (Fisons)

200ml Ultrapure water

Silver staining developer

7.5g Sodium hydroxide (Merck-BDH)

250ml Ultrapure water

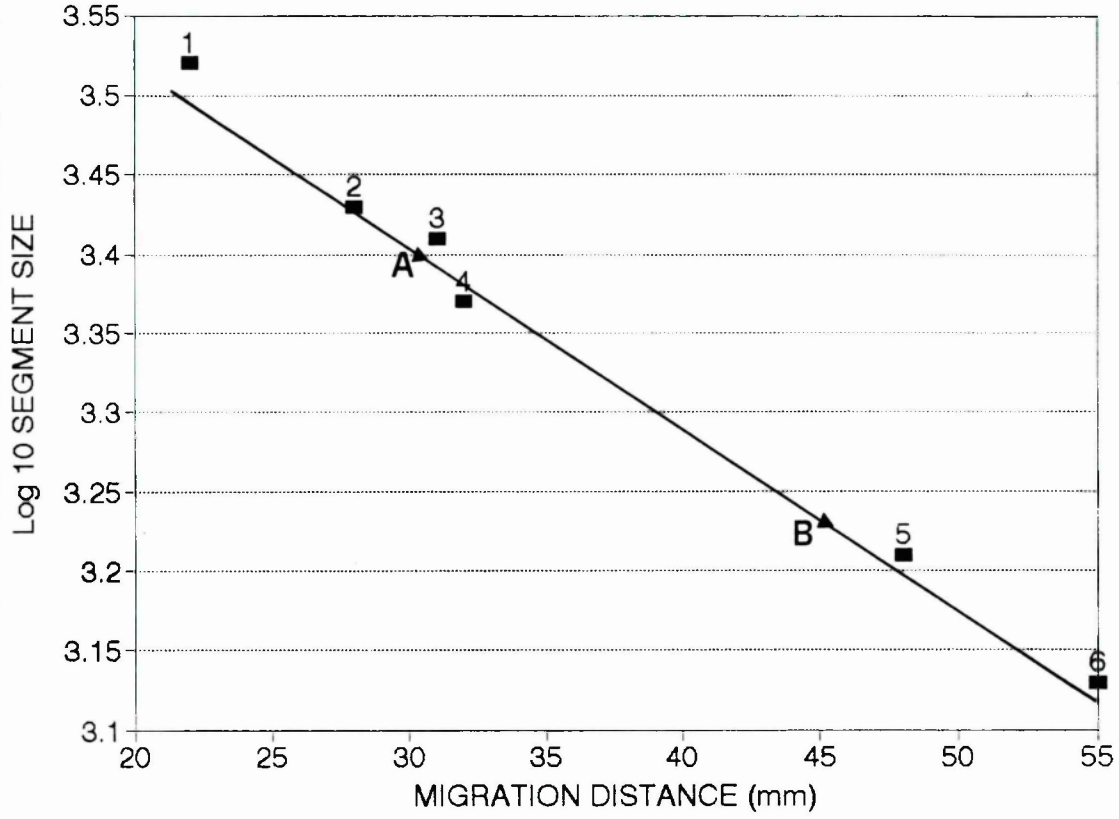
2ml 36% Formaldehyde solution (Merck-BDH)

A2) Preparation of size fractionated silica

Add 60g silicon dioxide, SiO_2; (Sigma) to demineralised water to a total volume of 500ml in a glass cylinder and allow the silica to sediment under gravity for 24h at room temperature. Extract 430ml of supernatant and add demineralised water to 500ml and shake vigorously and sediment again for 5h at room temperature. Extract 440ml of supernatant and add 600ul of HCL (32%, w/v) to adjust the silica suspension to pH 2, then aliquot then silica suspension in 4ml volumes into glass bijoux and sterilise by autoclaving.

A3)

Figure 69. Calibration curve for estimation of PBV and APBV segment sizes from the measurement of migration distance in a PAGE gel



1 to 6 are SA11 segments 1 to 6 (see R2)

A and B are segments 1 and 2 of a representative PBV (strain 21975/89)

A4) CsCl refractive index and buoyant density conversion tables

Table 28. Data for buoyant density determination taken from CsCl calibration curve (from CRC-Physical Chemistry, D-185)

Refractive Index (RI)	Buoyant Density (g/ml)
1.3620	1.3050
1.3630	1.3150
1.3640	1.3250
1.3650	1.3350
1.3660	1.3450
1.3670	1.3550
1.3680	1.3660
1.3690	1.3750
1.3700	1.3850
1.3710	1.3950
1.3720	1.4050
1.3730	1.4150
1.3740	1.4250
1.3750	1.4350
1.3760	1.4450
1.3770	1.4550

A5) Tissue culture media

a) EMEM maintenance medium

For 500ml,

450ml sterile water

50ml MEM with Earles salts (ICN)

13.5ml bicarbonate, 2g/l (ICN)

10ml penicillin (5000IU/ml) /streptomycin (5000ug/ml), (ICN)

5ml glutamine, 200mM (ICN)

5ml non-essential amino acids, 100X soln. (ICN)

5ml ampherotericin B, 250ug/ml (ICN)

b) HEK and MRC5 maintenance medium

10% 199 medium with Earles salts (ICN)

3.5% bicarbonate

0.1% gentamycin, 10mg/ml (ICN)

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PUBLICATIONS

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